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End organ damage in the metabolic syndrome and diabetes mellitus

Biochemical and magnetic resonance imaging studies

Nathanja Tjeerdema

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Biochemical and magnetic resonance imaging studies

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Because you're mine I walk the line

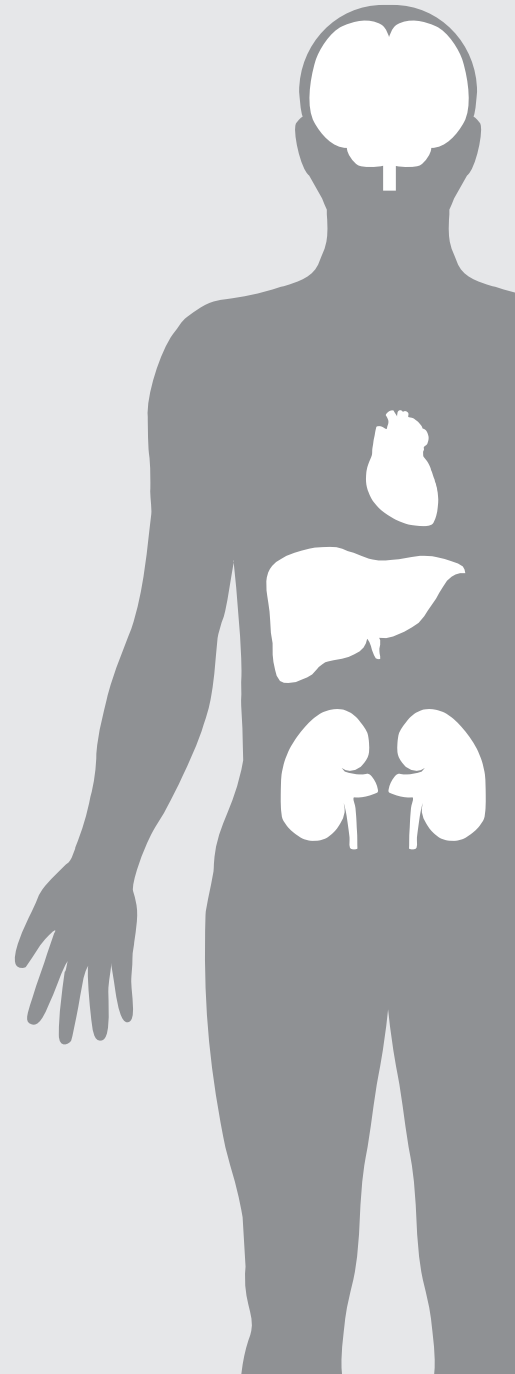
Johnny Cash

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Chapter 1

General introduction and outline



1.1 GENERAL INTRODUCTION

Cardiovascular disease (CVD) is the leading cause of death and illness in the Western world. In the Netherlands one out of four people dies from CVD (1) that includes myocardial infarction, congestive heart failure, stroke and peripheral artery disease. The major underlying pathogenic process of CVD is atherosclerosis, a progressive disease characterized by the accumulation of immune cells, lipids and fibrous elements in the large arteries (2,3). Advances in research suggest that the initiation and continuation of atherosclerotic diseases includes oxidative stress, endothelial dysfunction, inflammatory processes and vascular remodeling (4).

The increasing burden of CVD is in part driven by obesity. Obesity is defined as a body mass index [BMI; calculated as a person's bodyweight in kilograms divided by the square of his height in meters (kg/m^2)] as a BMI over 30. Worldwide the prevalence of obesity has more than doubled since 1980 and nowadays obesity-related mortality exceeds that caused by underweight (5). In the Netherlands half of the adults are overweight (6), which is defined as a BMI between 25 and 30. Large epidemiological studies show that the risk of coronary heart disease, ischemic stroke and type 2 diabetes mellitus increase steadily with an increasing body mass index (7). Especially the increase in visceral obesity as reflected by an increase in waist circumference seems to add to the cardiovascular risk (8,9) and correlates directly with metabolic dysfunction in epidemiological studies (10).

CVD in obesity

The mechanisms underlying CVD in obesity are complex and related to inflammation, insulin resistance, dyslipidemia, and hypertension which are closely interconnected. The clustering of these phenomena is called the metabolic syndrome. Despite the continuing debate regarding metabolic syndrome as a distinct disorder, it is a widely used term, as the clustering of abnormalities seems helpful in predicting CVD risk. The definition of the metabolic syndrome according to the International Diabetes Federation (IDF) criteria (11) includes two or more of the following criteria present in addition to increased abdominal obesity (waist circumference in males ≥ 94 cm, females ≥ 80 cm): hypertriglyceridemia (triglycerides (TG) ≥ 1.7 mmol/l or the use of lipid lowering medication); low levels of HDL-cholesterol (< 1.03 mmol/l in males and < 1.29 mmol/l in females); hyperglycemia (fasting glucose ≥ 5.6 mmol/l) or elevated blood pressure (systolic/diastolic blood pressure $\geq 130/\geq 85$ mmHg or the use of blood pressure lowering medication). Prevalence of the metabolic syndrome ranges from 20 to 30% of the adult population in Europe and varies somewhat depending on the age, geographic location or population studies (12).

Inflammation

Inflammation is considered a major pathogenic phenomenon in obesity related disorders. Indeed, inflammation is tightly connected to other phenomena of the metabolic syndrome as it may contribute to the pathogenesis of insulin resistance and dyslipidemia on the one hand, and plays a role in the adverse effects of insulin resistance, hyperglycemia and dyslipidemia on the other hand (13).

Inflammation in vascular injury and atherosclerosis

Atherosclerosis is a chronic inflammatory condition that involves infiltration and accumulation of cholesterol-rich atherogenic lipoproteins from the plasma into the intima of the vessel wall. This infiltration and accumulation of lipoproteins initiates a local inflammatory response (2,3) resulting in the activation of endothelial cells (14). In turn adhesion molecules such as vascular cell adhesion molecule (VCAM)-1, intercellular adhesion molecule (ICAM)-1 and E selectin are expressed. Adhesion molecules mediate the binding of circulating leukocytes to endothelial cells and their subsequent migration into the blood vessel wall, which is an important step in the initiation of atherosclerotic lesions (15). As monocytes migrate into the intima they proliferate and differentiate into macrophages that take up modified (e.g. oxidized) lipoproteins forming foam cells (16). These fat laden immune cells in the vascular wall excrete cytokines and chemokines that attract and activate additional immune cells.

Inflammation of adipose tissue and liver

In the metabolic syndrome, visceral adipose tissue is actively involved in inflammation. Lipid accumulation in adipocytes initiates a state of cellular stress and activation of the JNK(c-Jun N-terminal kinase) and IKK β (IkB kinase beta)/NF- κ B(nuclear factor kappa B) pathways. Macrophages are recruited and infiltrate the adipose tissue where they are the major source of several pro-inflammatory cytokines such as tumor necrosis factor (TNF)- α and interleukin (IL)-6 (17). Both JNK and IKK β /NF- κ B activated pathways in adipocytes and macrophages play important roles in inflammation-induced insulin resistance through inhibition of the insulin receptor signaling.

Cytokines as TNF- α and IL-6 stimulate the production of inflammatory acute-phase proteins by the liver such as C-reactive protein (CRP), a marker of inflammation and predictor of cardiovascular events (18,19). Similarly plasminogen activator inhibitor-1 (PAI-1) and fibrogen originate from hepatic tissue and are activators of the coagulation system, enhancing atherothrombosis (20-22).

In addition, the hepatocellular transcription factor NF- κ B, is activated in liver by high-fat diet, obesity and liver steatosis. NF- κ B activation results in hepatic production of pro-inflammatory cytokines (IL-6, IL-1 β and TNF- α as well as activation of Kupffer cells and macrophages) thereby contributing to a low grade systemic inflammatory state

(23). NF- κ B activation also results in hepatic and skeletal muscle insulin resistance. The concept that non-alcoholic fatty liver disease (NAFLD) may also drive the inflammatory cascade of atherosclerosis is now gaining acceptance (24).

Furthermore an increasing number of studies emphasize the central role of macrophage infiltration and foam cell formation in the pathogenesis of NASH, similar to the pathogenesis of atherosclerosis (25). The liver is thus a generator as well as a target of inflammatory factors. Increasing severity of NAFLD represents worsening of inflammatory and insulin resistant states with adverse cardiovascular outcomes.

Systemic inflammation

Countless epidemiologic studies have shown that low grade systemic inflammation, mostly reflected as elevated levels of CRP, is associated with metabolic syndrome, correlates with subclinical markers of atherosclerosis (26,27) and predicts future cardiovascular risk. This is generally attributed to the role of inflammation in plaque instability. Ridker et al. (28) were the first to report that elevated CRP levels predict future myocardial infarction and stroke. Although experimental studies have demonstrated that CRP is able to induce the expression of adhesion molecules, to activate endothelial cells and the complement system, CRP is generally regarded as a marker of inflammation that reflects the inflammatory status of the body, rather than an inflammatory mediator (29). In this view, the release of IL-1 β , IL-6 and TNF α , can stimulate the liver to produce CRP.

An important role for the innate immune system in the underlying mechanisms of systemic inflammation is suggested. Free fatty acids (FFAs) activate pattern recognition receptors (PRR) such as Toll-like receptor 4 (TLR4) that is predominantly expressed on immune cells like macrophages but have also been found in adipocytes (30,31) that are associated with inflammation. PRR induces NF- κ B and subsequently the secretion of pro-inflammatory cytokines.

Angiopoietin-like protein 4

Angiopoietin-like protein 4 (ANGPTL4) is a protein involved in a variety of functions including angiogenesis and energy metabolism. ANGPTL4 plays a role in plasma lipid metabolism by inhibiting the enzyme lipoprotein lipase (LPL). This results in suppression of the release of plasma TG-derived fatty acids and their subsequent uptake by tissues including adipose and skeletal muscle tissue. Recent studies suggest an additional role in inflammation. It was shown that the expression of ANGPTL4 is governed by the lipid-sensing peroxisome proliferator-activated receptors (PPARs) and is in vitro stimulated by FFAs (32-38). Because FFAs also activate PRR such as TLR4 in adipocytes and macrophages (39,40) it is likely that induction of inflammation (e.g. via TLR4 agonism) also increases ANGPTL4 expression.

ANGPTL4 as well as LPL are expressed at high levels in macrophages (41). Since foam cells are important in atherosclerosis (42) and given the facilitating role of LPL in macrophage foam cell formation (43,44), changes in ANGPTL4 expression may influence atherosclerosis development.

Insulin resistance

Development of decreased insulin sensitivity or insulin resistance is prevalent in obesity and overweight. Insulin resistance is characterized by reduced responsiveness of tissues to the effect of insulin. Insulin resistance often goes unnoticed and can be present many years before ultimately type 2 diabetes mellitus is diagnosed. Although often unnoticed the presence of insulin resistance independently predicts diabetes, hypertension and CVD. A validated and frequently used marker of insulin resistance is the Homeostasis Model Assessment Insulin Resistance (HOMA-IR). HOMA-IR is a model for assessing beta-cell function and incorporates both glucose and insulin concentrations and is calculated by the formula: (fasting insulin x fasting glucose)/22.5 or by the more recently updated computer model with normal insulin sensitivity defined as one (45). However no comparative data exist on the predictive effect of fasting plasma glucose levels, insulin levels and HOMA-IR on CVD.

High levels of insulin may independently contribute to CVD by stimulating proliferation of smooth muscle cells (46,47). However studies showing that insulin treatment is associated with a reduction of CVD events provide strong evidence against a direct atherogenic effect of insulin per se (48,49). This may be related to suppressed synthesis of proinflammatory cytokines in endothelial cells via nitric oxide mediated mechanisms. This beneficial effect may be decreased in insulin resistance (50). Furthermore insulin itself has an important role in hemodynamic regulation by modulating vascular tone probably also mediated by nitric oxide.

Insulin resistance may lead to hyperglycemia when pancreatic beta cells are no longer capable to compensate the increased hepatic glucose production and diminished uptake of glucose in peripheral tissues. Hyperglycemia in the context of insulin resistance may be directly atherogenic because it increases oxidative stress, thereby impairing endothelial function, which is implicated as one of the first steps in atherosclerosis. Moreover, during the exposure to hyperglycemia advanced glycation end products (AGES) are formed, resulting from advanced glycation of proteins and lipids, which contribute to a variety of microvascular and macrovascular complications through the creation of cross-links of collagen and by engaging the receptor for AGES in the vascular wall (51,52). Insulin resistance and the associated hyperglycemia thus may alter the mechanical properties of the arterial wall and on the long term cause increased arterial stiffness. However the effects of glucose and insulin on the arterial wall on the short term are unclear.

A third, possibly, most plausible way by which insulin resistance leads to CVD is via induction of dyslipidemia.

Dyslipidemia

When insulin resistance develops as a result of obesity, enlarged adipocytes of visceral adipose tissue release FFAs in the circulation due to increased lipolysis. These FFAs are subsequently transported to the liver, which leads to increased hepatic TG-rich very low-density lipoprotein (VLDL) production, one cause of the hypertriglyceridemia seen in insulin resistance. Besides the increased VLDL synthesis there is a decreased clearance of TG-rich lipoproteins in the circulation due to decreased lipoprotein lipase (LPL) activity. LPL is expressed by metabolic organs including the heart, skeletal muscle, white adipose tissue and brown adipose tissue. The enzyme is responsible for the hydrolysis of the TG from the core of VLDL into glycerol and fatty acids (53). Insulin is a potent stimulator of LPL but the responsiveness of LPL to insulin in obesity is diminished which is another cause of the hypertriglyceridemia found in insulin resistance.

The depletion of TG from VLDL particles by LPL results in the formation of VLDL remnants that are partly cleared by the liver. The remaining particles undergo further lipolysis to form low-density lipoproteins (LDL). In addition, both VLDL and LDL undergo exchange of TG for cholesteryl esters derived from high-density lipoprotein (HDL), a process mediated by cholesteryl ester transfer protein (CETP). CETP thus plays a pivotal role in the metabolism of VLDL and HDL by mediating the heteroexchange of cholesteryl esters in exchange for TG between HDL and (V)LDL. CETP activity is elevated in insulin resistance and thus further aggravates the dyslipidemia. The consequence of the action of CETP is the formation of small (dense) LDL particles and TG-enriched less stable HDL particles that are more rapidly catabolized by hepatic lipase, resulting in reduced HDL levels. HDL is generally believed to have anti-atherogenic properties due to its involvement in reverse cholesterol transport, its provision of antioxidant enzymes and its anti-inflammatory properties (21). Although liver and adipose tissue are the two major sources of circulating CETP in several mammalian species, the cellular origin of CETP is still uncertain.

Intracellular lipolysis also limits the capacity of adipose tissue to store TG. As a result transportation of FFAs towards other tissues that normally do not store lipids results in ectopic fat accumulation. Ectopic fat is associated with the generation of toxic intermediates of lipid oxygenation, mitochondrial dysfunction, activation of inflammatory pathways and may lead to cell death (54). Deposition of fat in the liver in the absence of alcohol intake or NAFLD aggravates hepatic insulin resistance due to decreased glucose uptake and decreased suppression of glucose production in the liver. NAFLD is the leading cause of chronic liver disease in the Western world and is present when there is evidence of hepatic steatosis either by imaging or by histology and there are no causes

for secondary hepatic fat accumulation (55). The estimated prevalence ranges from 30 to more than 60% in the metabolic syndrome (56,57). NAFLD represents a spectrum of liver disease ranging from steatosis through non-alcoholic steatohepatitis (NASH), characterized by a combination of fat accumulation and hepatic inflammation, and even further to fibrosis and cirrhosis. Interestingly, recent studies link NAFLD to CVD. In several studies NAFLD was strongly associated with high risk for atherosclerosis regardless of classical cardiovascular risk factors (24,58).

Hypertension

Besides insulin resistance, dyslipidemia and inflammation, overweight and obesity are often accompanied by high blood pressure. Hypertension is, in addition to aging, the major risk factor for CVD (59). Clinical studies have documented that weight gain can raise arterial pressure whereas weight loss has been shown successful in lowering blood pressure (60-62). Systematic reviews consistently report a decrease in systolic blood pressure of about 1 mmHg per kg weight loss (63). Elevated blood pressure is partly mediated through obesity-induced processes such as increased circulating plasma volume, activated rennin angiotensin system and increased sympathetic activity (64). The above described processes such as cross linking of collagen in insulin resistance and atherosclerotic plaque formation, lead to decreased arterial compliance and contribute to hypertension. In turn sustained high blood pressure itself gives continuous stress at the aortic wall which causes greater recruitment of inelastic collagen fibers and thereby a reduction in elasticity. In accordance studies show accelerated progression of arterial stiffness in (untreated) hypertension (65,66).

End organ damage

Cardiovascular complications are late events in a pathogenic process starting with endothelial dysfunction, oxidative stress and manifest atherosclerotic lesions which progress to organ damage and eventually result in organ failure. As CVD takes years to develop this allows time to early intervention in the chain of events precipitated by several risk factors. Generally adverse effects on the vascular tree are classified into macrovascular complications (coronary artery disease, peripheral arterial disease, stroke) and microvascular complications (nephropathy, neuropathy, retinopathy). A fifth of all cardiovascular events occur among individuals in whom traditional risk factors have not been identified (67,68). It is therefore important to search for asymptomatic organ damage before overt vascular complications develop. Biomarkers of end organ damage are characteristics that are objectively measured and evaluated as indicators of end organ damage. They may be either circulating substances, or characteristic alterations observed by imaging. Biomarkers may indicate underlying (subclinical) disease and/or predict future events beyond established major risk factors.

Arterial stiffness

Arterial stiffness in general, and aortic stiffness in particular, have become established as important biomarkers for CVD (69) and end organ damage as renal dysfunction, retinal damage and brain white matter injury (70,71). Most of the above described factors in the metabolic syndrome and diabetes directly or indirectly influence vascular wall properties leading to decreased arterial compliance.

Increased arterial stiffness has been found in the metabolic syndrome and diabetes and may be an important phenomenon linking obesity to increased cardiovascular risk. Large arteries play a key role in converting the pulsatile cardiac outflow produced during the ejection phase, into continuous blood flow throughout the arterial tree. By stretching the arterial wall, elastic energy accumulates that maintains the blood flow during diastole (Windkessel effect).

The damaging effect of high pulse pressure on kidney and brain is because cells depends on their resilience and are not protected by vasoconstricted arteries upstream as in the systemic circulation (72). Stiffness of the aorta may contribute to end-organ injury of the brain and kidney by exposing the small vessels to high pressure fluctuations and flow in the circulation (73). In addition arterial stiffening from aging or hypertension varies along the arterial tree, with increased stiffening of the central elastic arteries compared to more muscular distal arteries. Disproportional arterial stiffening may accelerate the transmission of excessive pulsatile energy toward the microcirculation.

Brain

Most studies regarding the brain are performed in type 2 diabetes and associations with cognitive dysfunction and structural brain changes (e.g. global brain atrophy and small-vessel disease) have been shown (74). In the last decades it has become increasingly clear that type 1 diabetes (T1DM) also affects the brain (75,76). Although adverse effects of diabetes on the brain have been reported as early as in the 1920's, for long research has mainly focused on cognitive function. More recently the application of magnetic resonance imaging of the brain has provided evidence for volume loss and subtle brain damage in T1DM. The high prevalence of white matter lesions, silent infarctions, microbleeds and early brain atrophy in T1DM (77-80) are related to cognitive decline and dementia. Although loss of cortical tissue has been demonstrated, less is known about possible atrophy of the subcortical grey matter structures. Volume loss of the hippocampus and left thalamus were only observed in small studies (81-84) and no data exist on subcortical structures. In addition to regional distribution of brain atrophy, connectivity between multiple brain areas is of interest as this is an important aspect of brain function related to cognitive function.

In addition to abnormalities in glucose metabolism a proposed underlying mechanism in the relationship between T1DM and the brain includes vascular disease. Arterial

stiffness in diabetes mellitus is increased and therefore it is an interesting marker with regard to brain injury as it reflects the cumulative influence of vascular risk factors with aging of the arterial wall.

Kidney

Renal function can be defined by estimated glomerular filtration rate (eGFR). Decreased eGFR, proteinuria and microalbuminuria are important markers of progressive chronic kidney disease and CVD. Hypertension is often accompanied with deterioration in renal function. Damage to the kidney due to chronic high blood pressure results from nephrosclerosis (thickening of the wall and the narrowing of the lumina of small arteries and arterioles). The severity of hypertensive renal damage depends on the degree to which renal autoregulatory mechanisms fail to prevent the blood pressure elevations from being transmitted to the vascular bed as is seen in diabetes.

Declined renal function in turn can lead to atherosclerosis and occurrence of vascular events via retention of sodium and water, increased levels of various atherogenic factors (enhanced oxidative stress and inflammation) and metabolic changes such as anaemia, hypocalcaemia, hypophosphatemia, hyperparathyroidism and vitamin D deficiency. Shared risk factors of declined renal function and hypertension including diabetes are often present. Both hypertension and chronic kidney disease can lead to arterial stiffness, however limited data are available on aortic stiffness in hypertension with normal renal function.

MR assessment of end organ damage

Advances in imaging techniques have resulted in imaging modalities for assessment of atherosclerosis and end organ damage at early stages of disease. Aortic stiffness can be accurately assessed by means of pulse wave velocity (PWV), which is considered the gold standard marker for vascular stiffness (85). In contrast to Doppler ultrasound, MRI enables regional assessment of PWV in multiple locations in the aorta and therefore allows the measurement of site-specific changes.

Furthermore MRI has been used to study the brain. Voxel based morphometry (VBM) is a novel and sophisticated analysis technique which allows testing for regional differences in grey and white matter density (86). Diffusion tensor imaging (DTI) is used for the measurement of structural connectivity (75). Based on difference in diffusivity, the integrity of white matter tracts can be assessed. White matter tracts provide connectivity between cortical areas and allow for communication between different brain regions.

1.2 OUTLINE OF THE THESIS

The studies in this thesis focus on biomarkers of cardiovascular end organ damage in the metabolic syndrome and diabetes.

The epidemic rise in overweight and obesity explains most of the increase in prevalence of type 2 diabetes thereby increasing the risk of CVD. Before diabetes is diagnosed, insulin resistance can be present for many years. As no comparative data exist on the effect of different components of insulin-resistance on cardiovascular events we performed a systematic review in **Chapter 2** and compared the associations of fasting glucose, fasting insulin and HOMA-IR with incident cardiovascular disease in adults without diabetes.

In **Chapter 3**, we investigated whether systemic inflammation is a determinant for plasma ANGPTL4 levels in patients with the metabolic syndrome and type 2 diabetes. To get more insight into the causal relationship between inflammation and ANGPTL4 expression we also assessed the effect of inflammatory stimuli on ANGPTL4 expression in vitro.

Aortic stiffness is a commonly used marker of vascular damage and can be assessed by means of pulse wave velocity (PWV). In **Chapter 4** we assessed the short-term effects of an oral glucose load on PWV. As the effect of insulin on vascular tone may be region-specific for various vascular beds and locations we developed an MRI protocol enabling regional assessment of the PWV in multiple locations or the aorta.

In **Chapter 5** we focused on the impact of type 1 diabetes mellitus on regional brain grey matter volume using voxel-based morphometry (VBM). Besides regional differences in brain volume, early detection of subtle deficits in microstructure by diffusion tensor imaging (DTI) (75) with MRI has become recently feasible. In **Chapter 6**, we assessed the possible association between aortic PWV and markers of microstructural brain damage i.e. brain white matter integrity in type 1 diabetes.

In **Chapter 7** we investigated the relation between aortic PWV and renal function in patients with hypertension. As arterial stiffening as well as impaired renal function often co-exist, it is not known whether increased PWV in patients with hypertension is directly related to high blood pressure or depends on co-existing renal dysfunction. Moreover no previous publications explored the possible relation with regional aortic stiffening. Therefore we assessed the relation between regional aortic PWV and performed this study in hypertensive patients with renal function in the normal range and compared them with healthy controls.

In addition to these studies on biomarkers of end organ damage, **Chapter 8** describes the result of an intervention study in a primary health care setting. Once subjects at high risk for cardiovascular disease are identified, adequate changes in lifestyle such as a more healthy diet, smoking prevention, increased physical activity and weight control can avoid over 75% of deaths (87). However changes in lifestyle are difficult to achieve and data on the large scale implementation in primary care are lacking. We therefore evaluated the feasibility and the effect of the implementation of a lifestyle intervention in subjects with the metabolic syndrome in a primary health care setting.

In **Chapter 9** the results of the studies described in this thesis are summarized and discussed.

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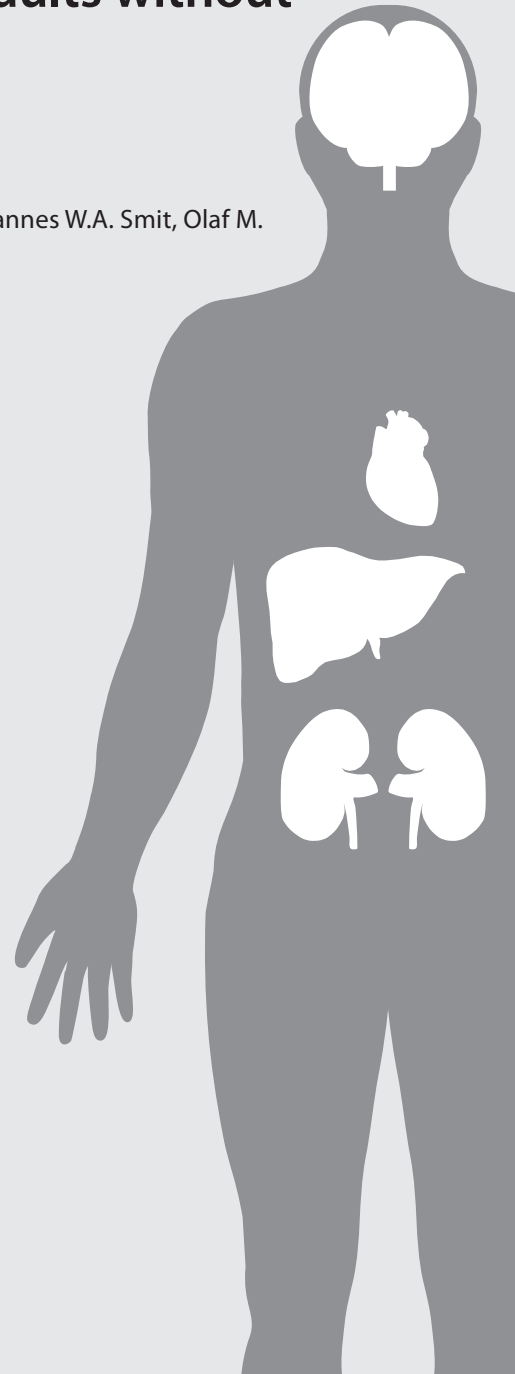
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Chapter 2

Insulin resistance and risk of incident cardiovascular events in adults without diabetes: meta-analysis

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ABSTRACT

Background

Glucose, insulin and Homeostasis Model Assessment Insulin Resistance (HOMA-IR) are markers of insulin resistance. The objective of this study is to compare fasting glucose, fasting insulin concentrations and HOMA-IR in strength of association with incident cardiovascular disease.

Methods

We searched the PubMed, MEDLINE, EMBASE, Web of Science, ScienceDirect and Cochrane Library databases from inception to March, 2011 and screened reference lists. Cohort studies or nested case-control studies that investigated the association between fasting glucose, fasting insulin or HOMA-IR and incident cardiovascular disease, were eligible. Two investigators independently performed the article selection, data extraction and risk of bias assessment. Cardiovascular endpoints were coronary heart disease (CHD), stroke or combined cardiovascular disease. We used fixed and random-effect meta-analyses to calculate the pooled relative risk for CHD, stroke and combined cardiovascular disease, comparing high to low concentrations of glucose, insulin or HOMA-IR. Study heterogeneity was calculated with the I^2 statistic. To enable a comparison between cardiovascular disease risks for glucose, insulin and HOMA-IR, we calculated pooled relative risks per increase of one standard deviation.

Results

We included 65 studies (involving 516,325 participants) in this meta-analysis. In a random-effect meta-analysis the pooled relative risk of CHD (95% CI; I^2) comparing high to low concentrations was 1.52 (1.31, 1.76; 62.4%) for glucose, 1.12 (0.92, 1.37; 41.0%) for insulin and 1.64 (1.35, 2.00; 0%) for HOMA-IR. The pooled relative risk of CHD per one standard deviation increase was 1.21 (1.13, 1.30; 64.9%) for glucose, 1.04 (0.96, 1.12; 43.0%) for insulin and 1.46 (1.26, 1.69; 0.0%) for HOMA-IR.

Conclusions

The relative risk of cardiovascular disease was higher for an increase of one standard deviation in HOMA-IR compared to an increase of one standard deviation in fasting glucose or fasting insulin concentration. It may be useful to add HOMA-IR to a cardiovascular risk prediction model.

INTRODUCTION

Cardiovascular disease is worldwide the leading cause of death [1]. Type 2 diabetes contributes importantly to cardiovascular disease, because it is highly prevalent and doubles cardiovascular disease risk [2,3]. Before type 2 diabetes is diagnosed, insulin resistance can be present for years, thereby increasing insulin and glucose concentrations [4,5].

Recent meta-analyses have shown that elevated insulin and glucose concentrations in persons without diabetes were associated with an increased cardiovascular disease risk [3,6]. In accordance, mechanistic studies have shown that elevated glucose and insulin concentrations can be pro-atherogenic [7,8]. Elevated insulin and glucose concentrations are direct consequences of insulin resistance. Insulin resistance can promote the development of atherosclerosis through elevated glucose and insulin concentrations, but also through mechanisms that involve dyslipidemia, hypertension, and inflammation [7,9]. Therefore, cardiovascular disease may be caused by insulin resistance rather than being a consequence of the toxic effects of elevated insulin or glucose concentrations. A validated and frequently used marker of insulin resistance is the Homeostasis Model Assessment Insulin Resistance (HOMA-IR). Since, HOMA-IR incorporates both glucose and insulin concentrations and represents insulin resistance, which can promote atherosclerosis through several mechanisms [7,9], it might be more strongly associated with cardiovascular disease than individual glucose or insulin concentrations. No meta-analysis thus far, has compared the strength of association between HOMA-IR and cardiovascular disease to associations between fasting glucose, fasting insulin and cardiovascular disease.

Our aim was to perform a systematic review and meta-analysis on the association between fasting glucose, fasting insulin, HOMA-IR and incident cardiovascular disease in individuals without diabetes. Our second aim was to compare fasting glucose, fasting insulin and HOMA-IR in strength of association with incident cardiovascular disease. We hypothesized that HOMA-IR is more strongly associated with incident cardiovascular disease than fasting glucose or fasting insulin.

METHODS

Data Sources and Searches

We searched the following databases from their inception to February 23, 2010: PubMed, MEDLINE, EMBASE, Web of Science, ScienceDirect and Cochrane Library. We updated the search to February 29th, 2011 for the MEDLINE and PubMed databases. The search strategy was optimized for all consulted databases, taking into account the differences

of the various controlled vocabularies as well as the differences of database-specific technical variations (e.g. the use of quotation marks). The reference lists of all potentially relevant articles were screened for additional publications. Detailed and database specific information about the search strategy is shown in **Table S1**.

Study Selection

The aim of our meta-analysis was to investigate the association between fasting glucose, fasting insulin, HOMA-IR and incident cardiovascular disease in individuals without diabetes at baseline. Cohort studies that measured glucose, insulin or HOMA-IR and reported original data on their association with cardiovascular disease, were eligible. We considered only cohort studies or nested case-control studies that measured glucose or insulin concentrations prior to the assessment of cardiovascular disease with a subsequent follow-up of minimally one year. No cross-sectional studies were eligible. In addition, articles in other languages than English were not eligible.

Since anti-diabetic drugs influence insulin and glucose concentrations, study populations should preferably have excluded participants with overt diabetes at baseline. However, population based studies that did not exclude participants with overt diabetes at baseline were eligible for inclusion. We excluded studies performed in populations exclusively consisting of persons with known diabetes or cohorts restricted to specific populations such as intensive care or transplant patients.

Studies that measured glucose or insulin concentrations in the fasting state were eligible for inclusion. Unfortunately, no uniform definition of fasting exists and many different definitions are being used [10]. Concentrations were considered to be fasting if study participants abstained from food for at least eight hours. Studies that reported the glucose or insulin concentrations to be fasting or measured after an overnight fast, but did not report the time span of fasting, were not excluded.

Studies reporting on at least one of the following endpoints were eligible: myocardial infarction, angina pectoris, stroke (ischemic or hemorrhagic), arrhythmias, congestive heart failure or sudden cardiac death separately or combinations. Studies that combined these endpoints with peripheral arterial disease, arterial aneurysm or arterial dissection in a composite endpoint were not excluded.

Furthermore, to be included studies should (1) report the association by comparing categories (percentiles or cut-off values), (2) express the association as relative risks (hazard ratios, rate ratios, risk ratios or odds ratios) with corresponding standard errors, confidence intervals or exact p-values and (3) adjust effect estimates at least for age and sex. In case of multiple publications arising from the same study population we included the study with the highest number of participants or the longest follow-up.

Data Extraction and Quality Assessment

Two investigators (K.G. and N.T.) independently performed the article selection based on titles and abstracts, data extraction and risk of bias assessment using a standard data sheet. Disagreement was resolved by consensus or by a third party (O.D.).

If necessary, glucose and insulin concentrations were recalculated to the international system of units (i.e. mmol/L for glucose and pmol/L for insulin) [11]. Values for HOMA-IR were based on values provided by the authors of included studies. In general, HOMA-IR is calculated by the formula: (fasting insulin x fasting glucose)/ 22.5 or by the more recently updated computer model [12]. We recalculated HOMA-IR values for studies that reported HOMA insulin sensitivity, which is the reciprocal of HOMA-IR.

We categorized study endpoints as (fatal or non-fatal): (1) coronary heart disease (CHD), (2) stroke and as (3) combined cardiovascular disease outcome (CVD), including studies contributing to 1 or 2. CHD was defined as myocardial infarction or angina pectoris; stroke consisted of hemorrhagic or ischemic stroke and CVD consisted of myocardial infarction, angina pectoris, hemorrhagic stroke, ischemic stroke, arrhythmias, congestive heart failure or sudden cardiac death.

Risk of bias assessment was based on design elements of cohort studies and nested case-control studies that could potentially bias the association between fasting glucose, fasting insulin, HOMA-IR and cardiovascular disease. Potential sources of bias were assessed by using a predefined assessment form. Dimensions considered for both cohort and nested case-control studies were (1) presence of overt diabetes at baseline, (2) presence of cardiovascular disease at baseline, (3) adequacy of exposure measurement, (4) missing glucose, insulin or HOMA-IR data, (5) adequacy of endpoint ascertainment. Bias was considered to be likely present when: (1) study populations had overt diabetes prevalence of twice their country specific diabetes prevalence estimates of 2011 [13]; indicating that studies have selected their study population based on high glucose concentrations (selection bias), (2) persons with prevalent cardiovascular disease according to their outcome definition were not excluded; (3) the time span of fasting was not reported, (4) $\geq 10\%$ missing data of the exposure except when data was missing completely at random (e.g. in the case of later introduction of the measurement), (5) outcome classification was based on self- or family reports, (6) there was $\geq 10\%$ loss to follow-up. Reliable methods of outcome assessment were assessment by medical records, death certificates or hospital discharge records. Diagnosis of myocardial infarction was considered reliable when WHO MONICA criteria or Minnesota coding of electrocardiograms during follow-up visits were used [14–16].

Data Synthesis and Analysis

Hazard ratios, rate ratios, risk ratios or odds ratios (relative risks) of cardiovascular disease comparing high to low concentrations of glucose, insulin or HOMA-IR values were

extracted. If necessary, we recalculated these relative risks in a way that the lowest category (percentile or cut-off value) comprised the reference category. Our first aim was to estimate the pooled relative risk for cardiovascular disease, when comparing categories (based on either percentiles or cut-offs) of high concentrations of glucose, insulin or HOMA-IR to categories of lower concentrations. We pooled maximally adjusted effect measures of studies with corresponding 95% confidence intervals (CI). For all analyses, both a fixed and a random-effect meta-analysis were performed. Study heterogeneity was calculated with the I^2 statistic. Elements of the risk of bias assessment were used to explore potential heterogeneity in sensitivity analyses. We assessed the presence of funnel plot asymmetry by calculating Egger's test [17].

Our second aim was to compare fasting glucose, fasting insulin and HOMA-IR in strength of association with cardiovascular disease by comparing pooled standardized relative risks (i.e. risk increase per increase of one standard deviation). First, we calculated the standard deviation per exposure by pooling reported standard deviations with a weight factor based on study size. Secondly, we applied the method of Hartemink et al. [18] to calculate an overall relative risk per one unit increase of the exposure. Then, we multiplied the logarithm of the relative risks by the pooled standard deviation of the exposure. In short, the method of Hartemink et al. [18] assumes a log-linear relation between the risk and the exposure. The input of the algorithm consists of the means and variances of the exposure within each category of the exposure, the log relative risks of the categories with respect to a reference category, and the number of cases within each category. To determine the category means and variances we applied various methods, depending on the kind of data reported in the article. We assumed a lognormal distribution for the exposures. Finally, we tested differences in pooled relative risks between the three exposures by using multivariate meta-analysis. Relative risks obtained from the same study (i.e. for studies that reported relative risks for more than one exposure) are likely to be correlated and this correlation is taken into account by multivariate meta-analysis.

We investigated sex differences in studies that presented sex-specific relative risks of cardiovascular disease by performing meta-analyses stratified by sex. Statistical analyses were performed with STATA Statistical Software (*Statacorp, College Station, Texas, USA*), version 11.2 and SAS software (*SAS Institute Inc., Cary, NC, USA*), version 9.2.

RESULTS

Search Results

We identified 4,792 unique publications by database search (MEDLINE: $n = 2,095$, PubMed: $n = 1,480$, EMBASE $n = 852$, Cochrane: $n = 112$, ScienceDirect: $n = 103$, Web of Science: $n = 86$) and by screening reference lists of potentially relevant articles ($n = 64$). After exclusion

of 4,469 publications by screening title and abstract, 323 publications were retrieved for detailed assessment of which 184 fulfilled inclusion criteria and were assessed in duplicate. To avoid multiple inclusions of the same study participants, we excluded 32 publications originating from the same study populations and included the publication with the largest population or the longest follow-up. Sixty-five studies (from 64 publications) were included. Forty-five studies presented data on fasting glucose, 17 studies presented data on fasting insulin and 16 studies presented data on HOMA-IR (**Figure 1**).

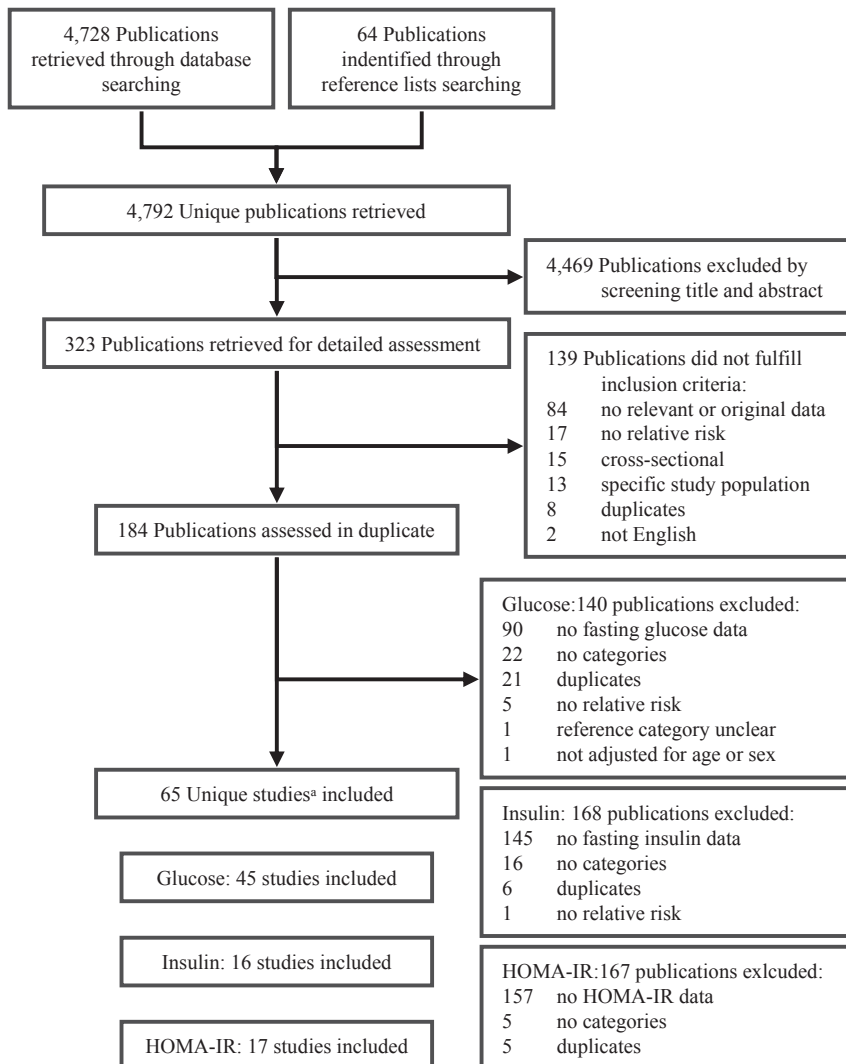


Figure 1. Summary of search results. ^a One publication consisted of two studies. HOMA-IR, Homeostasis Model Assessment insulin resistance

Study characteristics

Study characteristics of the included studies are summarized in **Table 1**. Sixty-four cohort studies and 1 nested case-control study were included. The controls in this case-cohort study were matched on time and therefore the odds ratio corresponds to a rate ratio [19]. Fifty-six studies presented a hazard ratio and nine studies presented an odds ratio. Most study populations consisted of both men and women. Individual study characteristics of included studies are shown in **Table S2**.

Table 1. Study characteristics of the included studies summarized for three exposures.

Characteristic	Exposure		
	Glucose (45 studies)	Insulin (16 studies)	HOMA-IR (17 studies)
Total participants	450,487	46,236	51,161
Participants per study (range)	541-63,443	541-13,446	839-6,942
Year of publication	1983-2010	1992-2010	2001-2010
Mean follow-up (years, range)	3.2-23.5	5.0-22.3 ^a	2.2-30
Study design			
Cohort	45	15	17
Nested case-control	0	1	0
CHD endpoint			
Number of studies	23	9	7
Events per study	23-4,490 ^b	16-677	33-169 ^b
Total events	10,884 ^b	2,149	441 ^b
Stroke endpoint			
Number of studies	14	2	4
Events per study	13-405 ^c	25-70	23-70 ^b
Total events	1,936 ^c	95	164 ^b
Combined CVD endpoint			
Number of studies	45	16	17
Events per study	23-4,490 ^b	16-492	58-340
Total events	19,993 ^b	3,329	3,035

Data are presented as number or range. ^a Three studies did not report follow-up time ^b Two studies did not report the number of participants who encountered the outcome of interest. ^c One study did not report the number of participants who encountered the outcome of interest. HOMA-IR, Homeostasis Model Assessment Insulin Resistance; CHD, coronary heart disease; CVD, cardiovascular disease

Risk of bias

The risk of bias assessment is summarized in **Table S3** and shown per study in **Table S4**. Most studies excluded persons with overt diabetes at baseline. One study included persons with prevalent cardiovascular disease and this was unclear in 20 studies. Twenty-two studies did not specify the time span of fasting or whether participants had

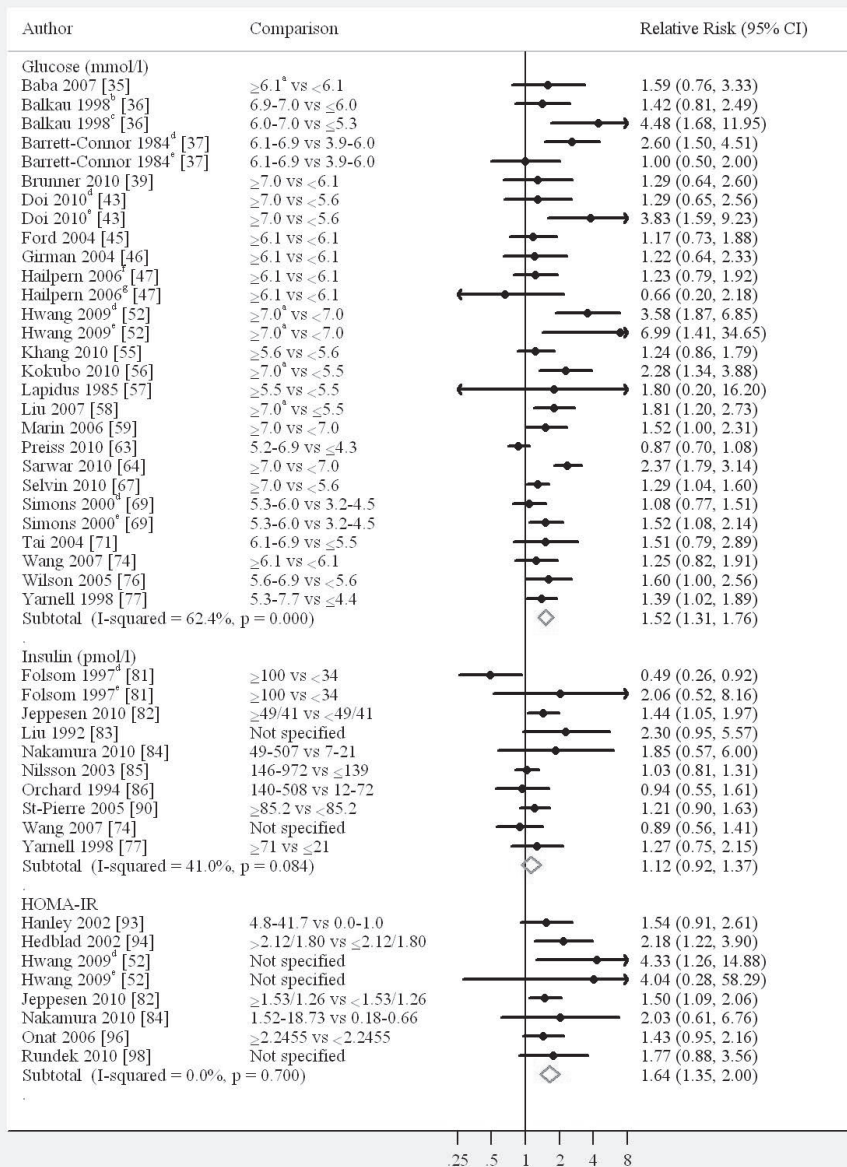


Figure 2. Random-effect meta-analyses of coronary heart disease risk for the highest category of glucose, insulin or HOMA-IR compared to the lowest category. ^a Or known diabetes was used to define the highest category. ^b Paris Prospective Study. ^c Helsinki Policemen Study. ^d Men. ^e Women. ^f Glomerular Filtration Rate ≥ 60 ml/min/1.73 m². ^g Glomerular Filtration Rate < 60 ml/min/1.73 m². 95% CI, 95% confidence interval; vs, versus; I-squared, measure of heterogeneity; HOMA-IR, Homeostasis Model Assessment Insulin Resistance

an overnight fast. Five studies had more than 10% missing data for glucose, four studies for insulin and three studies for HOMA-IR which was not reported to be completely at random. In 13 studies we considered bias to be likely present due to inadequate outcome assessment. The percentage of participants that were loss to follow-up ranged from 0% to 42%. Seven studies had a loss to follow-up of more than 10% and this was unclear in most studies. The p-values of Egger's test were 0.08 for glucose, <0.01 for insulin and <0.01 for HOMA-IR.

Comparison between glucose, insulin and HOMA-IR

In a random-effect meta-analysis the pooled relative risk of CHD comparing the highest versus the lowest category was 1.52 (95% CI: 1.31, 1.76; I^2 : 62.4%) for glucose, 1.12 (95% CI: 0.92, 1.37; I^2 : 41.0%) for insulin and 1.64 (95% CI: 1.35, 2.00; I^2 : 0%) for HOMA-IR (**Figure 2** and **Figure S1**). The pooled relative risks for the association with stroke and CVD, and meta-analyses stratified by sex for studies that provided sex-specific relative risks are summarized in **Figure S1**.

To enable a direct comparison between CHD and CVD risks for glucose, insulin and HOMA-IR we calculated pooled relative risks for an increase of one standard deviation [18]. We did not investigate the endpoint stroke, because only two studies investigated the association between insulin and stroke. The relative risks per increase of one standard deviation for glucose (1.05 mmol/L), insulin (43.53 pmol/L) and HOMA-IR (2.23 units) are shown in **Figure 3**. The pooled relative risk of CHD per one standard deviation increase was 1.21 (95% CI: 1.13, 1.30; I^2 : 64.9%) for glucose, 1.04 (95% CI: 0.96, 1.12; I^2 : 43.0%) for insulin and 1.46 (95% CI: 1.26, 1.69; I^2 : 0.0%) for HOMA-IR. The pooled relative risks of CHD for glucose, insulin, and HOMA-IR were all statistically different from each other (p-values: <0.05). The pooled relative risks of CVD for glucose, insulin, and HOMA-IR were not statistically different (p-value: 0.27).

Thirty-three studies provided sex-specific relative risks of CVD. Few studies provided relative risks of CHD or stroke for women and therefore we only investigated sex differences for incident CVD. Women had higher relative risks of CVD per one standard deviation increase of glucose (1.25 (95% CI: 1.11, 1.41; I^2 : 65.0%) versus 1.13 (95% CI: 1.08, 1.18; I^2 : 29.3%); p-value: 0.01) and insulin (1.24 (95% CI: 1.08, 1.44; I^2 : 18.5%) versus 1.06 (95% CI: 0.97, 1.16; I^2 : 60.4%); p-value: 0.03) and lower relative risk of CVD per one standard deviation increase of HOMA-IR (1.37 (95% CI: 1.05, 1.80; I^2 : 33.6%) versus 1.41 (95% CI: 1.12, 1.77; I^2 : 66.5%); p-value: 0.73) (**Figure 3**). In sensitivity analyses we excluded studies which had a high risk of bias based on items of the risk of bias assessment. The results of the meta-analyses were materially unchanged.

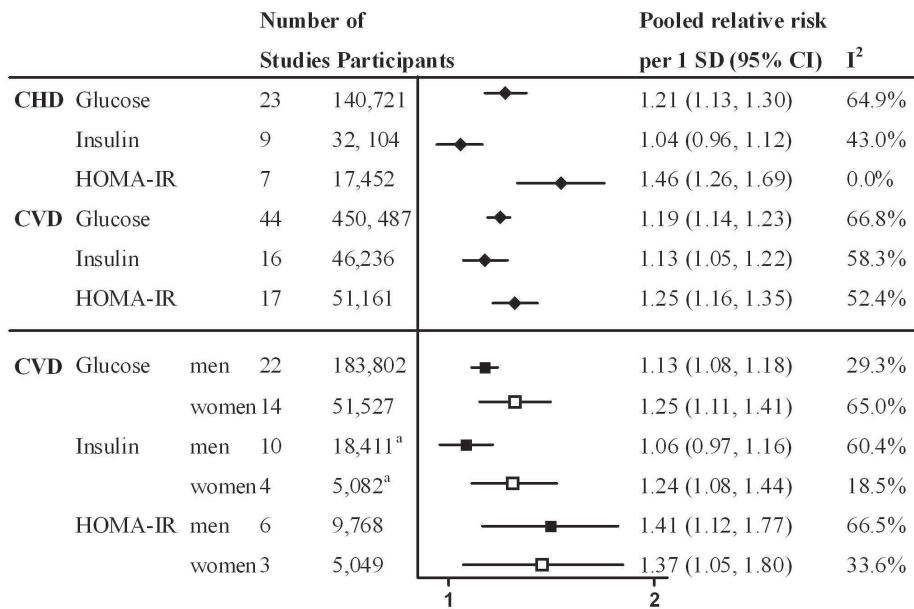


Figure 3. Results of random-effect meta-analyses comparing cardiovascular disease risk for an increase of one standard deviation. ^a 1 study did not specify sex-specific numbers. SD, standard deviation; 95% CI, 95% confidence interval; I², measure of heterogeneity; CHD, coronary heart disease and is defined as fatal or non-fatal myocardial infarction or angina pectoris; CVD, cardiovascular disease and is defined as myocardial infarction, angina pectoris, hemorrhagic stroke, ischemic stroke, arrhythmias, congestive heart failure or sudden cardiac death; HOMA-IR, Homeostasis Model Assessment Insulin Resistance

DISCUSSION

The present meta-analyses showed that fasting glucose, fasting insulin and HOMA-IR were all associated with incident cardiovascular disease in individuals without diabetes. In a standardized meta-analysis we found that coronary heart disease risk increased with 46% for an increase of one standard deviation in HOMA-IR concentration compared to an increase of 21% for fasting glucose concentration and an increase of 4% for fasting insulin concentration.

To our knowledge, this was the first meta-analysis that directly compared fasting glucose, fasting insulin and HOMA-IR in strength of association with cardiovascular disease.

A number of previous meta-analyses have investigated the association between fasting glucose, fasting insulin or HOMA-IR concentrations and cardiovascular disease by comparing high to low concentrations. Our pooled relative risks of cardiovascular disease (glucose: 1.44, insulin: 1.28, HOMA-IR: 1.44) are within the range of pooled relative risks reported in previous meta-analyses [6,20–22]. Differences in pooled relative risks between meta-analyses may be, for a large part attributed to different cut-off levels of

the exposure, leading to different causal contrasts. Further, differences in design aspects of meta-analyses may explain different pooled relative risks. For example, including studies with only fatal events versus studies with fatal and non-fatal events can result in different pooled RR for glucose, since diabetes seems to be a stronger risk factor for fatal than for non-fatal events [23]. Previous studies that investigated sex differences in the association between diabetes and cardiovascular disease found that women with diabetes had a higher relative risk than men with diabetes [3,24,25]. The pooled relative risks for an increase of one standard deviation in glucose and insulin were somewhat higher for women than for men, whereas there was less difference in relative risks between sexes for HOMA-IR. It has been proposed that diabetes may induce a more unfavorable cardiovascular risk profile in women than in men and thereby increases cardiovascular disease risk more in women [24,25]. Another explanation could be that these cardiovascular risk factors are not intermediates, but common causes of both diabetes and cardiovascular disease which may have a stronger effect in women than in men. However, most individual relative risks in this analysis were adjusted for cardiovascular risk factors. Leaving the possibility that there could still be residual confounding, for example by body composition and insulin resistance which are known to differ between men and women [26,27]. Even if relative risks are truly higher in women than in men, it is important to consider that absolute cardiovascular disease risk are lower [24]. In this meta-analysis, the relative risk of cardiovascular disease was higher for an increase of one standard deviation in HOMA-IR compared to an increase of one standard deviation in glucose or insulin. Animal studies have shown that insulin resistance plays an important role in the early and advanced stages of atherosclerosis, whereas hyperglycemia seems exclusively to be involved in early stages of atherosclerosis [9]. In addition, insulin resistance seems to modify the effect of insulin on the vascular wall; anti-atherogenic in the insulin sensitive state and pro-atherogenic in the insulin resistant state [8]. Unfortunately, it is not clear to what extent these pro-atherogenic mechanism contribute to the development of cardiovascular disease in humans.

A strength of this study is the large number of included studies comprising more than 500,000 participants. Therefore, the pooled effect estimates were not influenced largely by random error and it was possible to investigate different cardiovascular endpoints and sex differences. Secondly, in most studies we were able to calculate the relative risk for an increase of one standard deviation in the exposure. In this way, we adjusted for differences in assays and used cut-off points between studies and could compare the three exposures. Thirdly, we investigate the risk of incident coronary heart disease which is considered to be a homogeneous well-defined cardiovascular disease endpoint [28].

A general limitation of meta-analyses of observational studies is that the result may be a precise, but biased estimate. We assessed the risk of bias per study and performed sensitivity analyses excluding studies with a high risk of bias in a sensitivity analysis. This did

not change our results materially. We showed the presence of funnel-plot asymmetry by Egger's test. Sources of funnel plot asymmetry are publication bias, true heterogeneity of study effects or differences in study quality [17]. Since funnel-plot asymmetry was present for all three exposures, comparing three exposures still seems valid. Most studies included in our meta-analysis measured concentrations only once and are thereby susceptible to random measurement error. Random measurement error of the exposure leads to an attenuation of estimated effects [29]. Moreover, most studies only reported composite cardiovascular disease outcomes which may hamper a causal interpretation of reported risks if the exposure has no uniform effect on the different endpoints [30]. For example, elevated cholesterol concentration is a risk factor for coronary heart disease, but not for stroke [31,32]. Few studies reported stroke endpoints and associations in women; as a consequence the pooled relative risk of stroke for insulin was based on two studies and the pooled relative risk for HOMA-IR was based on four studies. Finally, we only included studies that measured HOMA-IR, which is a surrogate measure of insulin resistance and mainly reflects hepatic insulin resistance [12]. Therefore, it may not account for the total effect of insulin resistance. However, the application of the gold standard measurement, i.e. the euglycemic hyperinsulinemic clamp which is a measure of peripheral insulin resistance is often not feasible in large epidemiological studies.

More knowledge in the pathophysiology of atherosclerosis should guide type and initiation of treatment. For example, shifting the glucose distribution curve leftwards for the entire population as was postulated previously [33], is only effective when glucose itself is involved in atherosclerosis pathophysiology and when the intervention has a uniform effect in the entire population. However, the addition of HOMA-IR, a marker of insulin resistance to a risk prediction model may improve cardiovascular risk prediction. The addition of a fasting glucose measurement to the Framingham risk score resulted in a slight net reclassification improvement of 1.8% [34]. Whether the addition of HOMA-IR to a risk prediction model, on top of glucose, results in a more accurate reclassification of cardiovascular risk is unknown. Furthermore, this possible benefit should be carefully weighted against the extra costs involved with measuring both glucose and insulin. However, considering the addition of HOMA-IR to a prediction model is important, since many current models aiming to predict cardiovascular events are still not optimal to define high risk groups.

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APPENDIX

Table S1. Search strategy

MEDLINE (OVID-version)
(exp Insulin resistance/ OR insulin resistance.ti.ab OR insulin sensitivity.ti.ab OR hyperglycaemia*.ti.ab OR exp hyperglycemia/ OR hyperglycemia*.ti.ab OR exp hyperinsulinism/ OR hyperinsulin*.ti.ab OR glucose intolerance.ti.ab) AND (exp *Cardiovascular Diseases/ OR cardiovascular.ti OR myocardial.ti OR cerebrovascular.ti OR heart disease*.ti OR coronary artery disease*.ti OR stroke.ti OR coronary event*.ti OR cardiovascular mortality.ti OR cardiovascular event*.ti OR vascular disease*.ti) AND ((non-diabetic OR nondiabetic OR non-diabetes OR non-dm OR pre-diabetes OR prediabetes OR pre-diabetic OR pre-dm OR "subclinical diabetes" OR "subclinical diabetic" OR "sub-clinical diabetes" OR "sub-clinical diabetic" OR normal glucose tolerance OR ngt OR normoglycemia OR normoglycaemia OR normoglycemic OR normoglycaemic OR impaired glucose tolerance OR IGT OR "impaired fasting glucose" OR ifg OR without diabet* OR without diagnosed diabet*).mp OR ("free of".ti.ab ADJ10 diabetes.ti.ab) OR (without.mp ADJ5 metabolic syndrome.mp)) AND (exp Adult/ OR adult*.mp)
EMBASE (OVID-version)
(exp *Insulin resistance/ OR insulin resistance.ti OR exp *Insulin sensitivity/ OR insulin sensitivity.ti OR hyperglycaem*.ti OR exp *hyperglycemia/ OR hyperglycem*.ti OR exp *hyperinsulinism/ OR hyperinsulin*.ti OR exp *Hyperinsulinemia/ OR glucose intolerance.ti) AND (exp *Cardiovascular Disease/ OR cardiovascular.ti OR myocardial.ti OR cerebrovascular.ti OR heart disease*.ti OR coronary artery disease*.ti OR stroke.ti OR coronary event*.ti OR cardiovascular mortality.ti OR cardiovascular event*.ti OR vascular disease*.ti) AND ((non-diabetic OR nondiabetic OR non-diabetes OR non-dm OR pre-diabetes OR prediabetes OR pre-diabetic OR pre-dm OR "subclinical diabetes" OR "subclinical diabetic" OR "sub-clinical diabetes" OR "sub-clinical diabetic" OR normal glucose tolerance OR ngt OR normoglycemia OR normoglycaemia OR normoglycemic OR normoglycaemic OR impaired glucose tolerance OR IGT OR "impaired fasting glucose" OR ifg OR without diabet* OR without diagnosed diabet*).mp OR impaired glucose tolerance/ OR glucose blood level/) AND (exp Adult/ OR exp Aged/ OR exp Middle aged/ OR adult*.mp)
Web of Science
TI= ("Insulin resistance" OR "Insulin sensitivity" OR hyperglycaem* OR hyperglycem* OR hyperinsulin* OR "glucose intolerance") AND TI= ("Cardiovascular disease" OR "heart disease" OR "cardiac disease" OR "myocardial disease" OR "cerebrovascular disease" OR "coronary disease" OR "coronary artery disease" OR stroke OR "vascular disease"*) AND TS= (non-diabetic OR "pre-diabetes" OR "pre-diabetic" OR prediabetes OR prediabetic OR "subclinical diabetes" OR "sub-clinical diabetic" OR "sub-clinical diabetes" OR "normal glucose tolerance" OR ngt OR normoglycemi* OR normoglycaemi* OR "impaired glucose tolerance" OR ifg)
Science Direct
TITLE= ("Insulin resistance" OR "Insulin sensitivity" OR hyperglycaem* OR hyperglycem* OR hyperinsulin* OR "glucose intolerance") AND ("Cardiovascular disease" OR "heart disease" OR "cardiac disease" OR "myocardial disease" OR "cerebrovascular disease" OR "coronary disease" OR "coronary artery disease" OR stroke OR "vascular disease"*) AND ("non-diabetic" OR nondiabetic OR "non-diabetes" OR "non-dm" OR "pre-diabetes" OR prediabetes OR "pre-diabetic" OR "pre-dm" OR "subclinical diabetes" OR "sub-clinical diabetic" OR "sub-clinical diabetes" OR "normal glucose tolerance" OR ngt OR normoglycemi* OR normoglycaemi* OR "impaired glucose tolerance" OR IGT OR "impaired fasting glucose" OR ifg OR (without AND diabet*))

Cochrane library	
ID	Search
#1	MeSH descriptor Insulin Resistance explode all trees
#2	MeSH descriptor Hyperglycemia explode all trees
#3	MeSH descriptor Hyperinsulinism explode all trees
#4	(insulin resistance OR insulin sensitivity)ti
#5	(hyperglycaemi* OR hyperglycem* OR hyperinsulin* OR glucose intolerance)ti
#6	(hyperglycaemi* OR hyperglycem*)ti
#7	(hyperinsulin* OR glucose intolerance)ti
#8	(#1 OR #2 OR #3 OR #4 OR #5 OR #6 OR #7)
#9	MeSH descriptor Cardiovascular Diseases explode all trees
#10	(cardiovascular disease OR cardiovascular diseases OR myocardial disease OR myocardial diseases OR cerebrovascular disease OR cerebrovascular diseases OR heart disease OR heart diseases OR coronary artery disease OR coronary artery diseases OR stroke OR coronary event OR coronary events OR cardiovascular mortality OR cardiovascular event OR cardiovascular events OR vascular disease OR vascular diseases)ti
#11	(#8 AND (#9 OR #10))
#12	(non-diabetic OR nondiabetic OR non-dm OR pre-diabetes OR prediabetes OR pre-diabetic OR pre-dm OR "subclinical diabetes" OR "subclinical diabetic" OR "sub-clinical diabetes" OR "sub-clinical diabetic" OR normal glucose tolerance OR ngt OR normoglycemia OR normoglycemias OR normoglycaemias OR normoglycemic OR normoglycaemic OR impaired glucose tolerance OR IGT OR "impaired fasting glucose" OR ifg OR without diabet* OR without diagnosed diabet*)
#13	(#11 AND #12)

PubMed**Strategy 1:**

("Insulin resistance"[mesh] OR "insulin resistance"[tw] OR "insulin sensitivity"[tw] OR hyperglycaemia*[tw] OR hyperglycemia*[tw] OR "Hyperinsulinism"[mesh] OR hyperinsulin*[tw] OR "glucose intolerance"[tw]) AND ("Cardiovascular Diseases"[majr] OR cardiovascular[ti] OR myocardial[ti] OR cerebrovascular[ti] OR heart disease*[ti] OR coronary artery disease*[ti] OR stroke[ti] OR coronary event*[ti] OR cardiovascular mortality[ti] OR cardiovascular event*[ti] OR vascular disease*[ti] AND ("non-diabetic" OR nondiabetic OR "non-diabetes" OR "non-dm" OR "pre-diabetes" OR prediabetes OR "pre-diabetic" OR "pre-dm" OR "subclinical diabetes" OR "subclinical diabetic" OR "sub-clinical diabetes" OR "normal glucose tolerance" OR ngt[tw] OR normoglycemia OR normoglycaemia OR normoglycemias OR normoglycaemias OR normoglycemic OR normoglycaemic OR "impaired glucose tolerance" OR IGT[tw] OR "impaired fasting glucose" OR ifg OR "without diabetes" OR "prediabetic state"[mesh]) AND ("Adult"[mesh] OR adult*))

Strategy 2:

("Insulin resistance"[mesh] OR "insulin resistance"[tw] OR "insulin sensitivity"[tw] OR hyperglycaemia*[tw] OR hyperglycemia*[tw] OR "Hyperinsulinism"[mesh] OR hyperinsulin*[tw] OR "glucose intolerance"[tw]) AND ("Cardiovascular Diseases"[majr] OR cardiovascular[ti] OR myocardial[ti] OR cerebrovascular[ti] OR heart disease*[ti] OR coronary artery disease*[ti] OR stroke[ti] OR coronary event*[ti] OR cardiovascular mortality[ti] OR cardiovascular event*[ti] OR vascular disease*[ti] AND ("non-diabetic" OR nondiabetic OR "non-diabetes" OR "non-dm" OR "pre-diabetes" OR prediabetes OR "pre-diabetic" OR "pre-dm" OR "subclinical diabetes" OR "subclinical diabetic" OR "sub-clinical diabetes" OR "sub-clinical diabetic" OR "normal glucose tolerance" OR ngt[tw] OR normoglycemia OR normoglycaemia OR normoglycemias OR normoglycaemias OR normoglycemic OR normoglycaemic OR "impaired glucose tolerance" OR IGT[tw] OR "impaired fasting glucose" OR ifg OR "without diabetes" OR "prediabetic state"[mesh]) NOT ((animal NOT human) OR child OR children)

Strategy 3:

("Insulin resistance"[mesh] OR "insulin resistance"[tw] OR "insulin sensitivity"[tw] OR hyperglycaemia*[tw] OR hyperglycemia*[tw] OR "Hyperinsulinism"[mesh] OR hyperinsulin*[tw] OR "glucose intolerance"[tw] OR "Fasting plasma glucose" OR "Blood Glucose/metabolism"[mesh] OR "elevated glycated hemoglobin" OR "HbA(1c)" OR "Blood Glucose"[majr] OR "Blood Glucose/analysis"[mesh] OR "fasting glucose" OR "Hypoglycemic Agents/therapeutic use"[mesh] OR "Insulin/therapeutic use"[mesh] OR "glycated hemoglobin" OR "Hemoglobin A. Glycosylated/metabolism"[majr] OR "Insulin/blood"[majr] OR "Glucose/metabolism"[majr] OR glucometabolic[tw] OR "Blood Glucose"[mesh:noexpl] OR "blood glucose"[tiab]) AND ("Cardiovascular Diseases"[majr] OR cardiovascular[ti] OR myocardial[ti] OR cerebrovascular[ti] OR heart disease*[ti] OR coronary artery disease*[ti] OR stroke[ti] OR coronary event*[ti] OR cardiovascular mortality[ti] OR cardiovascular event*[ti] OR vascular disease*[ti] OR ("Coronary Disease"[mesh] AND ("non-diabetic"[ti] OR nondiabetic*[ti]) OR "Coronary Disease/epidemiology"[mesh]) AND ("non-diabetic" OR nondiabetic OR "non-diabetes" OR "non-dm" OR "pre-diabetes" OR prediabetes OR "pre-diabetic" OR "pre-dm" OR "subclinical diabetes" OR "sub-clinical diabetes" OR "normal glucose tolerance" OR ngt[tw] OR normoglycemia OR normoglycaemia OR normoglycemic OR normoglycaemic OR "impaired glucose tolerance" OR IGT[tw] OR "impaired fasting glucose" OR ifg OR "without diabetes" OR "prediabetic state"[mesh] OR ("Reference Values"[mesh] OR "Population Surveillance"[mesh] OR "Socioeconomic Factors"[mesh] OR "Disease-Free Survival"[mesh]) AND ("Heart Disease"[ti] OR "heart diseases"[ti] OR coronary[ti] OR chd[ti] OR "myocardial infarction"[ti] OR cardiovascular[ti]) OR nondiabetes OR "Comorbidity"[mesh] OR "serum insulin level" OR "serum insulin levels" OR "glucose tolerance"[ti] OR "non-diabetics" OR "initial glucose level" OR "initial glucose levels" OR "population studies"[ti] OR "body weight"[ti] OR predict[ti] OR predictor[ti] OR "non-insulin-resistant subjects" OR "all-causes"[ti] OR "excluding diabetes" OR ("control subjects" OR controls[tw]) AND ("metabolic syndrome"[ti] OR diabetes[ti] OR insulin[ti]) AND (cardiovascular[ti] OR coronary[ti] OR cardiac[ti] OR myocardial[ti]) OR "Population"[majr] OR "general population" OR "2-hour glucose" OR diabetes[ti] AND ("risk marker"[ti] OR "risk markers"[ti]) OR "population survey" OR "community-based sample" OR (Known[tiab] diabetes[tiab] excluded[tiab]) OR (excluding[tiab] diabetes[tiab] men[tiab] diabetes[tiab]) OR "whole population sample") AND ("Adult"[mesh] OR adult*))

Table S2. Characteristics of the included studies, organized by exposure

Source	Study design	Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a					End point	Events (n)	FU (yr) (mean)
								Smoking	BP	Lipids	Adiposity	CVD			
Glucose															
Baba, 2007 [35]	Cohort	2,024	38	Atomic bomb survivors	62.7 ^b	Cut-off	HR	Yes	No	No	No	Yes	Fatal & non-fatal CHD	49	U
Balkau, 1998 ^c [36]	Cohort	6,629	100	Civil servants	44-55	40-iles	HR	Yes	Yes	Yes	Yes	Yes	Fatal CVD Fatal CHD	446 284	20 ^d
Balkau, 1998 ^e [36]	Cohort	631	100	Policemen	44-55	40-iles	HR	Yes	Yes	Yes	Yes	Yes	Fatal Stroke Fatal CVD Fatal CHD	76 99 61	20 ^d
Barrett-Connor, 1984 ^f [37]	Cohort	1,610	100	Upper-middle class individuals	40-79	Cut-off	HR	Yes	Yes	Yes	Yes	No	Fatal Stroke Fatal CVD Fatal CHD	17 166 114	9 ^d
Barrett-Connor, 1984 ^g [37]	Cohort	2,015	0	Upper-middle class individuals	40-79	Cut-off	HR	Yes	Yes	Yes	Yes	No	Fatal CVD Fatal CHD	69 36	9 ^d
Bjornholt, 1999 [38]	Cohort	1,973	100	Employees	40-59	Quartiles	HR	U	U	U	U	U	Fatal CVD	242	22 ^d
Brunner, 2010 [39]	Cohort	6,868	67	Servants	40-55	Cut-off	HR	Yes	Yes	Yes	No	Yes	Fatal & non-fatal CHD	443	11.3
Cederberg, 2010 ^f [40]	Cohort	223	100	General population	60-64	Cut-off	OR	No	No	No	No	No	Fatal & non-fatal CVD	95	9.7
Cederberg, 2010 ^g [40]	Cohort	330	0	General population	60-64	Quintiles	OR	No	No	No	No	No	Fatal & non-fatal CVD	113	9.7
Chien, 2009 [41]	Cohort	16,590	60	Attendants health examination	51.9 ^b	Quintiles	HR	Yes	Yes	Yes	Yes	Yes	Fatal CVD	95	3.5 ^h
Dekker, 2005 ^f [42]	Cohort	615	100	General population	50-75	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	132	10 ^d
Dekker, 2005 ^g [42]	Cohort	749	0	General population	50-75	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	95	10 ^d

Table S2. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a				End point	Events (n)	FU (yr) (mean)
								BP	Lipids	Adi- posity	CVD			
Doi, 2010 ^f [43]	Cohort	1,037	100	General population	40-79	Cut-off	HR	Yes	Yes	Yes	Yes	Fatal & non-fatal CHD	75	14 ^d
								Yes				Fatal & non-fatal Stroke	61	
Doi, 2010 ^g [43]	Cohort	1,384	0	General population	40-79	Cut-off	HR	Yes	Yes	Yes	Yes	Fatal & non-fatal CHD	37	14 ^d
								Yes				Fatal & non-fatal Stroke	71	
Eberly, 2006 ^e [44]	Cohort	4,625	100	Trial participants with the MS	35-57	Cut-off	HR	Yes	Yes	Yes	Yes	Fatal CVD	899	18.4 ^h
Eberly, 2006 ^e [44]	Cohort	4,611	100	Trial participants without the MS	35-57	Cut-off	HR	Yes	Yes	Yes	Yes	Fatal CVD	846	18.4 ^h
Ford, 2004 [45]	Cohort	2,431	46	General population	30-75	Cut-off	HR	Yes	Yes	Yes	Yes	Fatal CVD	200	13.5
								Yes				Fatal CHD	147	
								Yes				Fatal Stroke	67	
Girman, 2004 [46]	Cohort	3,188	85	Trial participants	58 ^b	Cut-off	HR	No	No	No	No	Fatal & non-fatal CVD	U	5 ^d
								Yes				Fatal & non-fatal CVD	U	
								Yes				Fatal CHD	503 ⁱ	9.6
Halpern, 2006 ^k [47]	Cohort	9,918 ⁱ	62	Persons with untreated hypertension, GFR ≥ 60 ml/min/1.73 m ²	≥18	Cut-off	HR	Yes	Yes	Yes	Yes	Fatal CVD	337 ^j	9.6
								Yes				Fatal CHD	61 ⁱ	
								Yes				Fatal Stroke	61 ⁱ	
Halpern, 2006 ^m [47]	Cohort		62	Persons with untreated hypertension, GFR < 60 ml/min/1.73 m ²	≥18	Cut-off	HR	Yes	Yes	Yes	Yes	Fatal CVD	503 ⁱ	9.6
								Yes				Fatal CHD	337 ^j	
								Yes				Fatal Stroke	61 ⁱ	

Table S2. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Smoking	Adjustments ^a				End point	Events (n)	FU (yr) (mean)
									BP	Lipids	Adiposity	CVD			
Henry, 2002 [48]	Cohort	63,443	100	Attendants health examination	21-60	Cut-off	HR	Yes	Yes	Yes	Yes	No	Fatal CVD	171	8 ^m
Ho, 2008 [49]	Cohort	30,365	100	Attendants health examination	44 ^b	Cut-off	HR	No	Yes	Yes	Yes	No	Fatal CVD	527	13.6 ^h
Hsu, 2007 [50]	Cohort	4,888	100	General population	≥30	Cut-off	HR	Yes	No	No	No	No	Fatal CVD	246 ⁱ	10.6
Hsu, 2007 [50]	Cohort	6,170	0	General population	≥30	Cut-off	HR	Yes	No	No	No	No	Fatal CVD	246 ⁱ	10.6
Hunt, 2004 [51]	Cohort	2,617	44	General population	25-64	Cut-off	HR	No	No	No	No	No	Fatal CVD	84	12.7
Hwang, 2009 ^f [52]	Cohort	1,761	100	Volunteers health care program	20-78	Cut-off	OR	Yes	No	Yes	No	No	Fatal & non-fatal CVD	106	8.7
													Fatal & non-fatal CHD	39	
													Fatal & non-fatal Stroke	70	
Hwang, 2009 ^g [52]	Cohort	674	0	Volunteers health care program	20-78	Cut-off	OR	Yes	No	Yes	No	No	Fatal & non-fatal CVD	30	8.7
													Fatal & non-fatal CHD	8	
													Fatal & non-fatal Stroke	23	
Jeppesen, 2007 [53]	Cohort	2,493	49	General population	41-71	Cut-off	HR	Yes	No	Yes	No	No	Fatal & non-fatal CVD	233	9.4 ^h
Juutilainen, 2006 ^f [54]	Cohort	574	100	General population	45-64	Cut-off	HR	Yes	No	Yes	No	No	Fatal CVD	75	18 ^d
Juutilainen, 2006 ^g [54]	Cohort	707	0	General population	45-64	Cut-off	HR	Yes	No	Yes	No	No	Fatal CVD	23	18 ^d

Table 52. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a				End point	Events (n)	FU (yr) (mean)
								Smoking	BP	Lipids	Adiposity	CVD		
Khang, 2010 ^c [55]	Cohort	9,791	45	General population	≥20	Cut-off	HR	No	Yes	Yes	Yes	No	Fatal & non-fatal CVD	288 122 184
Kokubo, 2010 ^c [56]	Cohort	5,321	47	General population	30-79	Cut-off	HR	Yes	Yes	Yes	Yes	No	Fatal & non-fatal CVD	364 166 198 11.7 ^h
													Fatal & non-fatal CVD	
													Fatal & non-fatal CVD	
													Fatal & non-fatal CVD	
Lapidus, 1985 [57]	Cohort	1,424	0	General population	38-60	Cut-off	OR	No	No	No	No	No	Fatal & non-fatal CVD	23 13 12 ^d
													Fatal & non-fatal CVD	
													Fatal & non-fatal CVD	
													Fatal & non-fatal CVD	
Liu, 2007 [58]	Cohort	30,378	53	General population	35-64	Cut-off	HR	Yes	No	Yes	No	No	Fatal & non-fatal CVD	738 U U 10 ^d
													Fatal & non-fatal CVD	
													Fatal & non-fatal CVD	
													Fatal & non-fatal CVD	
Marin, 2006 ^c [59]	Cohort	4,434	62	Patients from primary care centers	25-99	Cut-off	HR	Yes	Yes	Yes	Yes	No	Fatal & non-fatal CVD	147 5.0
													Fatal & non-fatal CVD	
Nakanishi, 2004 [60]	Cohort	6,182	100	Employees government	35-39	Cut-off	HR	Yes	No	No	No	No	Non-fatal CVD	7 ^d
Nichols, 2009 [61]	Cohort	20,226	48	Health insurance members with NGT or IGT	64	Cut-off	HR	Yes	Yes	Yes	Yes	Yes	(Fatal?) & non-fatal CVD	6.6

Table 52. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a					End point	Events (n)	FU (yr) (mean)
								Smoking	BP	Lipids	Adiposity	CVD			
Nilsson, 2007 [62]	Cohort	5,047	40	General population	46-68	Cut-off	HR	No	No	No	No	No	Fatal & non-fatal CVD	176	10.7
Preiss, 2010 [63]	Cohort	6,447	100	Trial participants	45-64	Quintiles	HR	Yes	No	Yes	Yes	Yes	Fatal & non-fatal CVD	2,381	15 ^d
													Fatal CVD	1,474	
													Fatal & non-fatal CHD	405	
Sarwar, 2010 [64]	Cohort	18,333	49	General population	32-61	Cut-off	HR	Yes	Yes	Yes	Yes	No	Fatal Stroke	4,490	23.5
													Fatal & non-fatal CHD		
													Fatal CVD	434	
Sattar, 2008 [65]	Cohort	3,361	47	Trial participants	70-82	Cut-off	HR	No	No	No	No	Yes	Fatal & non-fatal CVD	162	4.1
Schillaci, 2004 [66]	Cohort	1,742	55	Patients with hypertension	50.3	Cut-off	HR	Yes	Yes	Yes	No	No	Fatal & non-fatal CVD	1,198	14 ^h
Selvin, 2010 [67]	Cohort	11,092	42	General population	47-69	Cut-off	HR	Yes	Yes	Yes	Yes	No	Fatal CHD	358	
Shin, 2009 ^e [68]	Cohort	57,237	57	Attendants health examination	40-89	Cut-off	HR	Yes	Yes	Yes	No	No	Fatal Stroke	129	5.6
													Fatal CVD		
Simons, 2000 ^f [69]	Cohort	1,045	100	General population	60-79	Quartiles	HR	U	U	U	U	U	Fatal & non-fatal CHD	339	9.4 ^h
													Fatal & non-fatal Stroke	139	
Simons, 2000 ^g [69]	Cohort	1,374	0	General population	60-79	Quartiles	HR	U	U	U	U	U	Fatal & non-fatal CHD	339	9.4 ^h
													Fatal & non-fatal Stroke	141	
Smith, 2002 [70]	Cohort	4,014	40	General population	45-64	Quintiles	HR	Yes	Yes	Yes	Yes	No	Fatal & non-fatal Stroke	764	8.5 ^h
													Fatal & non-fatal CVD		

Table 52. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Men Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a					End point	Events (n)	FU (yr) (mean)
								Smoking	BP	Lipids	Adiposity	CVD			
Tai, 2004 [71]	Cohort	5,091	50	General population and volunteers following health intervention program	U	Cut-off	HR	No	No	No	No	No	Fatal & non-fatal CHD	128	8 ^d
Thomas, 2007 ^e [72]	Cohort	2,863	50	General population	25-74	Cut-off	HR	Yes	No	No	No	No	Fatal CVD	30	8.5
Tsai, 2008 [73]	Cohort	35,259	66	Attendants health examination	≥40	Cut-off	HR	No	No	No	No	No	Fatal CVD	468	15 ^h
Wang, 2007 [74]	Cohort	541	50	Participants with a high risk for DM	≥25	Cut-off	OR	Yes	No	Yes	No	No	Non-fatal CHD	236	5 ^d
Watanabe, 2008 [75]	Cohort	28,449	34	Attendants health examination	≥20	Cut-off	HR	No	No	No	No	No	Non-fatal CVD	265	4.5
Wilson, 2005 [76]	Cohort	3,323	47	Offspring Framingham Study	22-81	Cut-off	HR	No	No	No	No	No	Fatal & non-fatal CVD	174	8 ^p
Yarnell, 1998 [77]	Cohort	4,197	100	General population	45-63	Quintiles	OR	Yes	Yes	Yes	No	Yes	Fatal & non-fatal CHD	492	9.7 ^d
Zhang, 2009 [78]	Cohort	2,173	45	General population	≥45	Cut-off	HR	Yes	No	No	No	No	Fatal & non-fatal Stroke	52	4.6 ^h
Insulin															
Bonara, 2007 [79]	Cohort	839	50	General population	40-79	Quartiles	HR	Yes	Yes	Yes	Yes	No	Fatal & non-fatal CVD	118	15 ^d
Chien, 2008 [80]	Cohort	2,165	44	General population	≥35	Quartiles	HR	Yes	No	No	Yes	No	Fatal & non-fatal CHD	166	10.5 ^h
Dekker, 2005 ^f [42]	Cohort	615	100	General population	50-75	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	132	10 ^d

Table S2. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a						End point	Events (n)	FU (yr) (mean)
								Smoking	BP	Lipids	Adiposity	CVD	Other			
Dekker, 2005 ⁹ [42]	Cohort	749	0	General population	50-75	Quartiles	HR	No	No	No	No	No	No	Fatal & non-fatal CVD	95	10 ^d
Folsom, 1997 ⁷ [81]	Cohort	13,446 ¹	100	General population	45-64	Cut-off	HR	Yes	Yes	Yes	Yes	No	Yes	Fatal & non-fatal CHD	209	U
Folsom, 1997 ⁹ [81]	Cohort	13,446 ¹	0	General population	45-64	Cut-off	HR	Yes	Yes	Yes	Yes	No	Yes	Fatal & non-fatal CHD	96	U
Jeppesen, 2010 [82]	Cohort	2,265	49	General population	41-71	Quartiles	HR	Yes	Yes	Yes	No	No	No	Fatal CVD Fatal & non-fatal CHD	119 169	12.6 ^h
Juutilanen, 2006 ^f [54]	Cohort	574	100	General population	45-64	Quartiles	HR	Yes	No	Yes	No	No	Yes	Fatal CVD	75	18 ^d
Juutilanen, 2006 ^g [54]	Cohort	707	0	General population	45-64	Quartiles	HR	Yes	No	Yes	No	No	Yes	Fatal CVD	23	18 ^d
Liu, 1992 [83]	Cohort	589	35	General population	≥25	Deciles	HR	No	No	No	No	No	No	Non-fatal CHD	16	6.7
Nakamura, 2010 [84]	Cohort	2,548	100	General population	35-59	Quartiles	HR	Yes	Yes	Yes	No	No	Yes	Fatal & non-fatal CVD Fatal & non-fatal CHD Fatal & non-fatal CHD	58 33 25	11
Nilsson, 2003 [85]	Cohort	6,074	100	General population	25-64	Deciles	HR	Yes	Yes	Yes	Yes	No	Yes	Fatal & non-fatal Stroke	677	19
Orchard, 1994 [86]	Nested case-control	622	100	Men with high risk of CVD	35-57	Quartiles	OR	Yes	Yes	Yes	No	No	Yes	Fatal & non-fatal CHD	208	U
Oterdoom, 2009 ^f [87]	Cohort	3,290	100	General population	28-75	Quartiles	HR	No	No	No	No	No	No	Fatal & non-fatal CVD	242	7.5 ^d

Table S2. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a					End point	Events (n)	FU (yr) (mean)
								Smoking	BP	Lipids	Adiposity	CVD			
Oterdoorn, 2009 ^b [87]	Cohort	3,626	0	General population	28-75	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	98	7.5 ^d
Pyorala, 1998 [88]	Cohort	970	100	Policemen	34-64	Quintiles	HR	Yes	Yes	No	Yes	No	Fatal & non-fatal stroke	70	22.3 ^h
Rutter, 2005 [89]	Cohort	2,898	45	Offspring Framingham study	26-82	Quartiles	HR	Yes	No	Yes	Yes	No	Fatal & non-fatal CVD	186	6.7 ^h
St-Pierre, 2005 [90]	Cohort	1,824	100	General population	34-64	Quartiles	HR	Yes	No	No	No	No	Fatal & non-fatal CHD	284	13 ^d
Wang, 2007 [74]	Cohort	541	50	Participants with a high risk of DM	≥25	Quartiles	OR	Yes	No	Yes	No	No	Non-fatal CHD	236	5 ^d
Yarnell, 1998 [77]	Cohort	1,896	100	General population	45-63	Quintiles	OR	Yes	Yes	Yes	No	Yes	Fatal & non-fatal CHD	221	9.7 ^d
HOMA-IR															
Arnlov, 2010 [91]	Cohort	958	100	General population	50	Quartiles	HR	Yes	No	Yes	No	No	Fatal & non-fatal CVD	318	30 ^h
Barr, 2010 [92]	Cohort	6,942	55	General population	≥25	Quintiles	HR	Yes	Yes	Yes	Yes	Yes	Fatal & non-fatal CVD	225	5 ^h
Bonora, 2007 [79]	Cohort	839	50	General population	40-79	Quartiles	HR	Yes	Yes	Yes	Yes	No	Fatal & non-fatal CVD	118	15 ^d
Chien, 2008 [80]	Cohort	2,165	44	General population	≥35	Quartiles	HR	Yes	No	No	Yes	No	Fatal & non-fatal CHD	166	10.5 ^h
Dekker, 2005 ^f [42]	Cohort	615	100	General population	50-75	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	132	10 ^d
Dekker, 2005 ^g [42]	Cohort	749	0	General population	50-75	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	95	10 ^d

Table 52. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a				Other	End point	Events (n)	FU (yr) (mean)	
								Smoking	BP	Lipids	Adiposity					CVD
Hanley, 2002 [93]	Cohort	2,413	43	General population	25-64	Quintiles	OR	Yes	Yes	Yes	Yes	No	Yes	Fatal & non-fatal CVD	187	7.5 ^h
														Fatal & non-fatal CHD	U	
														Fatal & non-fatal CHD	U	
Hedblad, 2002 [94]	Cohort	4,748	39	General population	46-68	Quartiles	HR	Yes	Yes	Yes	Yes	No	Yes	Fatal & non-fatal Stroke	62	5 ^h
Hwang, 2009 ^f [52]	Cohort	1,761	100	Volunteers health care program	20-78	Tertiles	OR	Yes	No	Yes	No	No	No	Fatal & non-fatal CVD	106	8.7
														Fatal & non-fatal CHD	39	
														Fatal & non-fatal CHD	70	
Hwang, 2009 ^g [52]	Cohort	674	0	Volunteers health care program	20-78	Tertiles	OR	Yes	No	Yes	No	No	No	Fatal & non-fatal Stroke	30	8.7
														Fatal & non-fatal CVD	8	
														Fatal & non-fatal CHD	23	
Isomaa, 2001 [95]	Cohort	4,483	48	Families	35-70	Quartiles	OR	Yes	No	Yes	No	No	No	Fatal CVD	209	6.9 ^h
Jeppesen, 2010 [82]	Cohort	2,265	49	General population	41-71	Quartiles	HR	Yes	Yes	Yes	No	No	No	Fatal CVD	119	12.6 ^h
														Fatal & non-fatal CHD	169	
Nakamura, 2010 [84]	Cohort	2,548	100	Factory workers	35-59	Quartiles	HR	Yes	Yes	Yes	No	No	Yes	Fatal & non-fatal CVD	58	11
														Fatal & non-fatal CHD	33	
														Fatal & non-fatal CHD	25	
														Fatal & non-fatal Stroke		

Table S2. Characteristics of the included studies, organized by exposure (continued)

Source	Study design	Men Number	Men (%)	Study population	Age (range)	Categories	Effect measure	Adjustments ^a					End point	Events (n)	FU (yr) (mean)
								Smoking	BP	Lipids	Adiposity	CVD			
Nilsson, 2007 [62]	Cohort	5,047	40	General population	46-68	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	176	10.7
Onat, 2006 [96]	Cohort	1,348	44	General population	52.2 ^b	Quartiles	OR	No	No	Yes	Yes	No	Fatal & non-fatal CHD	147	2.2
Oterdoom, 2009 ^f [87]	Cohort	3,290	100	General population	28-75	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	242	7.5 ^d
Oterdoom, 2009 ^g [87]	Cohort	3,626	0	General population	28-75	Quartiles	HR	No	No	No	No	No	Fatal & non-fatal CVD	98	7.5 ^d
Resnick, 2003 [97]	Cohort	2,283	43	American Indians from 12 tribes	45-74	Tertiles	HR	Yes	Yes	Yes	Yes	No	Fatal & non-fatal CVD	181	7.6
Rundek, 2010 [98]	Cohort	1,509	36	General population	≥39	Quartiles	HR	Yes	Yes	Yes	Yes	No	Fatal & non-fatal CVD	180	8.5
													Fatal CVD	45	
													Fatal & non-fatal CHD	46	
													Fatal & non-fatal Stroke		
Rutter, 2005 [89]	Cohort	2,898	45	Offspring Framingham study	22-81	Quartiles	HR	Yes	No	Yes	No	No	Fatal & non-fatal CVD	186	6.7 ^h

^a All studies are at least adjusted for age and sex. ^b Mean. ^c Paris Prospective Study. ^d Unspecified. ^e Helsinki Policemen Study. ^f Analyses stratified by sex; men. ^g Analyses stratified by sex; women. ^h Median. ⁱ Analyses stratified by the presence of the metabolic syndrome; with the metabolic syndrome. ^j Analyses stratified by the presence of the metabolic syndrome; without the metabolic syndrome. ^k Analyses stratified by glomerular filtration rate (GFR); GFR ≥ 60 ml/min/1.73 m². ^l Analyses stratified, number represents total for both groups. ^m Analyses stratified by glomerular filtration rate (GFR); GFR < 60 ml/min/1.73 m². ⁿ Minimum. ^o Studies provided additional data stratified by sex. ^p maximum

BP, blood pressure; CVD, (pre-existing) cardiovascular disease; FU, follow-up; HR, hazard ratio; CHD, coronary heart disease; U, unclear; OR, odds ratio; MS, Metabolic Syndrome; GFR, glomerular filtration rate; NGT, normal glucose tolerance; IGT, impaired glucose tolerance; DM, diabetes mellitus

Table S3. Risk of bias assessment summarized for three exposures

Potential sources of bias	Exposure		
	Glucose (45 studies)	Insulin (16 studies)	HOMA-IR (17 studies)
Oversampling of overt diabetes at baseline			
No	87%	100%	94%
Yes	0%	0%	0%
Unclear	13%	0%	6%
Presence of outcome at baseline			
No	71%	75%	44%
Yes	2%	0%	0%
Unclear	27%	25%	56%
Unspecified definition of fasting			
No	62%	75%	82%
Yes	38%	25%	18%
More than 10% missing data for the exposure			
No	25%	56%	47%
Yes	11%	25%	18%
Unclear	64%	19%	35%
Unreliable outcome assessment			
No	73%	50%	65%
Yes	16%	31%	24%
Unclear	11%	19%	11%
More than 10% loss to follow-up			
No	31%	31%	17%
Yes	9%	13%	24%
Unclear	60%	56%	59%

HOMA-IR: Homeostasis Model Assessment Insulin Resistance

Table S4. Risk of bias assessment categorized per exposure and per study.

Source	Overt DM at baseline (%) (CSPE)	Presence of outcome at baseline (%)	Unspecified definition of fasting	Missing data for the exposure (%)	Endpoint assessment	Loss to follow-up (%)
Glucose						
Baba, 2007 [35]	Unclear (11%)	0%	Unclear	Unclear	Self-reports, electrocardiograms, medical records, death certificates	Unclear
Balkau, 1998 ^a [36]	0% (7%)	0%	Overnight	Unclear	Administrative departments employer, relatives, medical records	5%
Balkau, 1998 ^b [36]	0% (9%)	0%	12 hours	Unclear	Death certificates, autopsy reports, medical records	0%
Barrett-Connor, 1984 [37]	0% ^c (11%)	0%	12 hours	6%	Death certificates	0%
Bjornholt, 1999 [38]	0% ^d (6%)	0%	12 hours	Unclear	Death certificates	Unclear
Brunner, 2010 [39]	0% (7%)	0%	Unclear	5%	Medical records, death certificates	Unclear
Cederberg, 2010 [40]	0% (9%)	Unclear	12 hours	Unclear	Hospital discharge diagnoses, death certificates	Unclear
Chien, 2009 [41]	0% ^d (10%)	0%	Unclear	Unclear	Death certificates	Unclear
Dekker, 2005 [42]	0% ^c (7%)	Unclear	Overnight	3%	Medical records, fatal events: unclear	25%
Doi, 2010 [43]	♂:15% ^c (11%) ♀:10% ^c	0%	Overnight	4%	Clinical examinations, autopsies	0%
Eberly, 2006 [44]	Unclear (11%)	0%	12 hours	1%	Death certificates	Unclear
Ford, 2004 [45]	3% (11%)	0%	10 hours	74%	Death certificates	2%
Girman, 2004 [46]	0% ^c (11%)	Unclear	Unclear	Unclear	Unclear	Unclear
Hailpern, 2006 [47]	0% ^c (11%)	0%	Unclear	Unclear	Death certificates	Unclear
Henry, 2002 [48]	0% (7%)	0%	Unclear	Unclear	Death certificates	Unclear
Ho, 2008 [49]	5% (11%)	0%	Unclear	Unclear	Death certificates	Unclear
Hsu, 2008 [50]	8% ^d (10%)	0%	Overnight	Unclear	Death certificates	Unclear
Hunt, 2004 [51]	0% ^c (11%)	0%	12 hours	4%	Death certificates	1%
Hwang, 2009 [52]	7% ^c (9%)	Unclear	Overnight	30%	Self-reports and medical records, fatal events: unclear	Unclear
Jeppesen, 2007 [53]	3% (8%)	0%	Overnight	Unclear	Register of hospitalizations, death certificates	Unclear

Table S4. Risk of bias assessment categorized per exposure and per study (continued).

Source	Overt DM at baseline (%) (CSPE)	Presence of outcome at baseline (%)	Unspecified definition of fasting (%)	Missing data for the exposure (%)	Endpoint assessment	Loss to follow-up (%)
Juutilainen, 2006 [54]	0% (9%)	0%	12 hours	Unclear	Death certificates	Unclear
Khang, 2010 [55]	0% (9%)	0%	Unclear	13%	Hospital admissions and discharges, death certificates	Unclear
Kokubo, 2010 [56]	Unclear (11%)	Unclear	Unclear	3%	Medical records, death certificates	10%
Lapidus, 1985 [57]	1% (6%)	0%	Overnight	Unclear	Medical records, death certificates	3%
Liu, 2007 [58]	7% ^c (9%)	Unclear	Unclear	Unclear	WHO MONICA protocol	14%
Marin, 2006 [59]	11% ^c (8%)	0%	10 hours	Unclear	Medical records, death certificates	2%
Nakanishi, 2004 [60]	0% ^d (11%)	Unclear	8 hours	Unclear	Medical record, fatal events: unclear	3%
Nichols, 2009 [61]	0% ^c (11%)	0%	Unclear	Unclear	Medical record, fatal events: unclear	Unclear
Nilsson, 2007 [62]	0% ^c (6%)	0%	Overnight	Unclear	Hospital discharge diagnoses	Unclear
Preiss, 2010 [63]	0% ^c (7%)	Unclear	Unclear	Unclear	Hospital discharge diagnoses, death certificates	Unclear
Sarwar, 2010 [64]	0% ^c (7%)	0%	8 hours	Unclear	Central registries, death certificates	1%
Sattar, 2008 [65]	0% ^c (7%)	0%	Unclear	Unclear	Review board, death certificates	Unclear
Schillaci, 2004 [66]	6% ^c (8%)	0%	Unclear	Unclear	Medical records, fatal events: unclear	1%
Selvin, 2010 [67]	0% (11%)	Unclear	12 hours	Unclear	Medical records, death certificates	Unclear
Shin, 2009 [68]	6% (9%)	0%	10 hours	0%	Death certificates	0%
Simons, 2000 [69]	0% ^c (8%)	20%	12 hours	Unclear	Register of hospitalizations, death certificates	2%
Smith, 2002 [70]	2% (11%)	Unclear	8 hours	5%	Medical records, death certificates	Unclear
Tai, 2004 [71]	0% ^c (11%)	0%	10 hours	Unclear	Mycardial register, hospital discharge register, death certificates	Unclear
Thomas, 2007 [72]	Unclear (9%)	0%	12 hours	Unclear	Death certificates	Unclear
Tsai, 2008 [73]	Unclear (10%)	0%	Unclear	3%	Death certificates	Unclear
Wang, 2007 [74]	0% ^c (9%)	0%	Overnight	37%	Medical records (only non-fatal events)	42%
Watanabe, 2008 [75]	12% (11%)	0%	Overnight	Unclear	Elektrocardiograms at follow-up examination (only non-fatal events)	Unclear

Table S4. Risk of bias assessment categorized per exposure and per study (continued).

Source	Overt DM at baseline (%) (CSPE)	Presence of outcome at baseline (%)	Unspecified definition of fasting (%)	Missing data for the exposure (%)	Endpoint assessment	Loss to follow-up (%)
Wilson, 2005 [76]	0% ^c (11%)	Unclear	Unclear	Unclear	Clinical examinations, physician outpatient record, hospitalizations	Unclear
Yarnell, 1998 [77]	0% (7%)	Unclear	Overnight	5%	Self-reports, electrocardiograms, hospital activity analysis, death certificates	6%
Zhang, 2009 [78]	Unclear (9%)	0%	Unclear	17%	Unclear	Unclear
Insulin						
Bonora, 2007 [79]	7% ^c (8%)	0%	Overnight	31%	Medical records, death certificates	0%
Chien, 2008 [80]	7% (10%)	0%	12 hours	Unclear	Self-reports and medical records, fatal events: unclear	Unclear
Dekker, 2005 [42]	0% ^c (7%)	Unclear	Overnight	3%	Medical records, fatal events: unclear	25%
Folsom, 1997 [81]	0% ^c (11%)	0%	12 hours	15%	Hospital discharge diagnoses, electrocardiograms at follow-up examination, death certificates, relatives, interviews physicians	Unclear
Jeppesen, 2010 [82]	0% ^c (8%)	0%	Overnight	1%	Death certificates	Unclear
Juutilainen, 2006 [54]	0% (9%)	0%	12 hours	Unclear	Death certificates	Unclear
Liu, 1992 [83]	0% ^c (11%)	0%	Unclear	37%	Electrocardiograms at follow-up examination (only non-fatal events)	Unclear
Nakamura, 2010 [84]	0% ^c (11%)	Unclear	Unclear	1%	Medical records, fatal events: unclear	1%
Nilsson, 2003 [85]	0% ^c (6%)	0%	Unclear	10%	Malmö heart register, death certificates	Unclear
Orchard, 1994 [86]	0% (11%)	0%	Unclear	6%	Medical records, electrocardiograms at follow-up examination, death certificates	Unclear
Oterdoom, 2009 [87]	0% ^c (7%)	Unclear	8 hours	2%	Hospital discharge diagnosis, death certificates	Unclear
Pyorala, 1998 [88]	0% ^d (9%)	0%	12 hours	2%	Medical records, death certificates, autopsy reports	0%
Rutter, 2005 [89]	0% ^c (11%)	0%	Overnight	3%	Review board	Unclear
St-Pierre, 2005 [90]	0% (11%)	0%	12 hours	Unclear	Death certificates, non-fatal events: unclear	0%
Wang, 2007 [74]	0% ^c (9%)	0%	Overnight	37%	Medical records (only non-fatal events)	42%
Yarnell, 1998 [77]	0% (7%)	Unclear	Overnight	5%	Self-reports, electrocardiograms, hospital activity analysis, death certificates	6%

Table S4. Risk of bias assessment categorized per exposure and per study (continued).

Source	Overt DM at baseline (%) (CSPE)	Presence of outcome at baseline (%)	Unspecified definition of fasting exposure (%)	Missing data for the exposure (%)	Endpoint assessment	Loss to follow-up (%)
HOMA-IR						
Arnlov, 2010 [91]	0% ^c (6%)	Unclear	Overnight	Unclear	Hospital discharge register, medial records for heart failure diagnosis, death certificates	0%
Barr, 2010 [92]	0% ^c (8%)	Unclear	9 hours	1%	Medical records, death certificates	19%
Bonora, 2007 [79]	0% ^c (8%)	0%	Overnight	31%	Medical records, death certificates	0%
Chien, 2008 [80]	7% (10%)	0%	12 hours	Unclear	Self-reports, medical records, fatal events: unclear	Unclear
Dekker, 2005 [42]	0% ^c (7%)	Unclear	Overnight	3%	Medical records, fatal events: unclear	25%
Hanley, 2002 [93]	0% (11%)	Unclear	12 hours	Unclear	Self-reports, death certificates	35%
Hedblad, 2002 [94]	0% (6%)	0%	Overnight	4%	Malmö Heart register, death certificates	Unclear
Hwang, 2009 [52]	7% ^c (9%)	Unclear	Overnight	30%	Self-reports, medical records, fatal events: unclear	Unclear
Isomaa, 2001 [95]	Unclear (9%)	Unclear	Unclear	Unclear	Death certificates	20%
Jeppesen, 2010 [82]	0% (8%)	0%	Overnight	1%	Death certificates	Unclear
Nakamura, 2010 [84]	0% (11%)	Unclear	Unclear	1%	Medical records: fatal events: unclear	1%
Nilsson, 2007 [62]	0% (6%)	0%	Overnight	Unclear	Hospital discharge diagnosis	Unclear
Onat, 2006 [96]	0% ^d (7%)	Unclear	11 hours	6%	Self-reports, electrocardiograms, fatal events: unclear	Unclear
Oterdoom, 2009 [87]	0% (7%)	Unclear	8 hours	2%	Hospital discharge diagnoses, death certificates	Unclear
Resnick, 2003 [97]	0% (11%)	Unclear	Unclear	4%	Medical records, hospital discharge diagnosis	Unclear
Rundek, 2010 [98]	0% (11%)	0%	12 hours	44%	Death certificates, medical records	Unclear
Rutter, 2005 [89]	0% (11%)	0%	Overnight	Unclear	Review board	Unclear

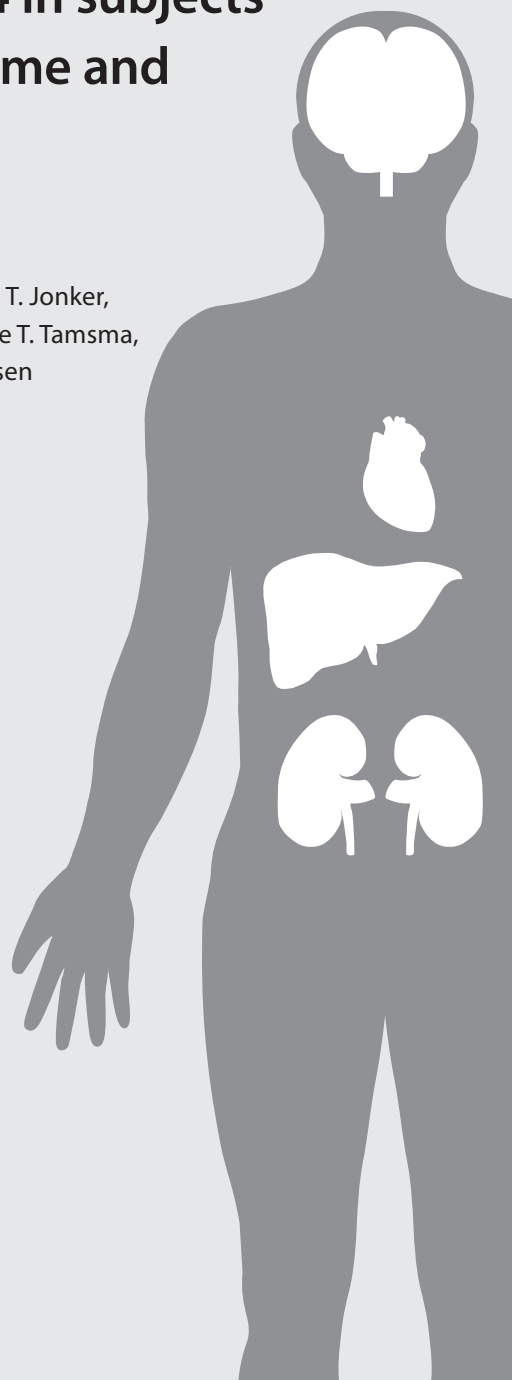
^a Paris Prospective Study. ^b Helsinki Policemen Study. ^c Percentage includes newly diagnosed diabetes. ^d Percentage includes known diabetes and newly diagnosed diabetes. References are listed in References S5. DM, diabetes mellitus; CSPE, country specific prevalence estimates; HOMA-IR, Homeostasis Model Assessment Insulin Resistance

Chapter 3

Inflammation increases plasma angiotensin-like protein 4 in subjects with the metabolic syndrome and type 2 diabetes

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ABSTRACT

Background

Angiopoietin-like protein 4 (ANGPTL4) inhibits lipoprotein lipase and associates with dyslipidemia. The expression of ANGPTL4 is regulated by free fatty acids (FFA) that activate lipid-sensing peroxisome proliferator-activated receptors (PPARs), but FFA can also activate pattern recognition receptors including Toll-like receptor 4 (TLR4) in macrophages.

Objective

To assess whether systemic low grade inflammation is a determinant for plasma ANGPTL4 levels in patients with the metabolic syndrome (MetS) and type 2 diabetes mellitus (T2DM).

Design

We studied 335 male subjects: healthy controls (Controls), subjects with the metabolic syndrome without inflammation (MetS-I) and with low grade inflammation (MetS+I), and T2DM patients. All non-diabetic subjects included in the present study were initially matched for waist circumference. In plasma, ANGPTL4, C-reactive protein (CRP) and metabolic parameters were determined. Underlying mechanisms were examined using human macrophages *in vitro*.

Results

As compared to Controls, plasma ANGPTL4 levels were increased in subjects with MetS-I, MetS+I and T2DM. Furthermore ANGPTL4 was increased in T2DM compared to MetS-I. In fact, plasma CRP correlated positively with plasma ANGPTL4. *In vitro* studies showed that Toll-like receptor 3/4 activation largely increased the expression and release of ANGPTL4 by macrophages.

Conclusions

Plasma ANGPTL4 levels in humans are predicted by CRP, a marker of inflammation, and ANGPTL4 expression by macrophages is increased by inflammatory stimuli.

INTRODUCTION

The angiotensin-like proteins are a family of secreted proteins that play an important role in energy metabolism. Angiotensin-like protein 4 (ANGPTL4) is expressed in numerous cell types including adipocytes, hepatocytes, (cardio)myocytes, endothelial cells and macrophages (1,2). ANGPTL4 plays a role in plasma lipid metabolism by inhibiting the enzyme lipoprotein lipase (LPL). This action of ANGPTL4 results in suppression of the release of plasma triglyceride-derived fatty acids and their subsequent uptake by underlying metabolic tissues including adipose tissue, skeletal muscle and the heart, with concomitant hypertriglyceridemia (3-5).

Expression of ANGPTL4 in a variety of tissues, including adipose tissue and liver, is governed by the lipid-sensing peroxisome proliferator-activated receptors (PPARs) α , β and γ , and is stimulated *in vitro* by free fatty acids (FFA) (1,6-8). Accordingly, dietary modulations that increase plasma FFA levels, including prolonged fasting, very-low calorie diet, and high-fat, high-energy diet, also increase plasma ANGPTL4 levels, most likely via activation of PPARs in tissues such as adipose tissue and liver (9,10). Besides activating PPARs, there is compelling evidence that FFA also activate pattern recognition receptors (PRRs) in adipocytes and macrophages, including the Toll-like receptor 4 (TLR4) (11,12). Accordingly, it is of interest to study the impact of inflammation and (associated) TLR activation on ANGPTL4 expression.

The metabolic syndrome is a progressive inflammatory disease, ranging from mild dyslipidemia and impaired fasting glucose levels to full blown type 2 diabetes mellitus (T2DM) (13,14). Interestingly, the progression of metabolic syndrome (MetS) towards T2DM is accompanied by progressive inflammation, which may result in increased plasma levels of ANGPTL4 that, in its turn, could aggravate dyslipidemia. Therefore, the aim of the present study was to assess whether systemic low grade inflammation is a determinant for plasma ANGPTL4 levels in patients with the MetS and T2DM. To get more insight into the causal relationship between inflammation and ANGPTL4 expression we assessed whether inflammatory stimuli increase ANGPTL4 expression *in vitro*.

RESEARCH DESIGN AND METHODS

Human studies

Subjects

To assess the effects of low grade systemic inflammation on plasma ANGPTL4 levels we studied four groups of human subjects: healthy controls (Controls, n=95), subjects with metabolic syndrome but *without* systemic inflammation (MetS-I, n=106), subjects with

metabolic syndrome accompanied by low grade systemic inflammation (MetS+I, n=66), and patients with T2DM (T2DM, n=68). All non-diabetic subjects included in the present study were initially matched for waist circumference (maximum difference 17 cm) and age (maximum difference 19 years). The metabolic syndrome was defined according to the IDF criteria (15) i.e. two or more of the following criteria present in addition to increased waist circumference (male ≥ 94): triglycerides ≥ 1.7 mmol/l (≥ 150 mg/dl) / the use of lipid lowering medication, HDL cholesterol < 1.03 mmol/l (< 40 mg/dl) in men and < 1.29 mmol/l (< 50 mg/dl) in women, fasting glucose ≥ 5.6 mmol/l (100 mg/dl) or BP $\geq 130/\geq 85$ mmHg / the use of blood pressure lowering medication. Low grade inflammation was defined as moderately elevated CRP: $3.0 \text{ mg/l} \leq \text{CRP} < 15.0 \text{ mg/l}$.

Controls (n=95), subjects with MetS-I (n=66; 62% of all included subjects with MetS-I) and subjects with MetS+I (n=33; 50% of all included subjects with MetS+I) were selected from a survey in the general population in the city of Rijswijk, The Netherlands. For this survey, all non-diabetic subjects between 40-70 years of age of four general practitioners were invited for a cardiovascular risk screening (n=2942). Subjects with diagnosed diabetes, known terminal disease and a history of psychiatric disorders or substance abuse were excluded. Screening was performed on 2079 subjects (response rate of 71%) and CRP was determined for 1515 subjects. After excluding subjects with high CRP (defined as $> 15 \text{ mg/l}$, n=45), subjects with missing values (n=13), female subjects (n=792) and subjects with cardiovascular disease (n=55), 610 male subjects were eligible. Only male subjects were selected to avoid the potential interference of sex steroid hormones on study parameters. The metabolic syndrome was present in 44% (n=268) of the 610 males. A total of 98 controls and 102 subjects with metabolic syndrome were selected for the current study. In addition 1 subject with new diagnosed diabetes, 1 subject with a very high level of ANGPTL4 and four subjects with missing data (n=3 Control and n=1 MetS) were excluded therefore leading to 95 controls and 99 MetS subjects. Subjects with metabolic syndrome were divided in MetS-I (n=66) and MetS+I (n=33).

Subjects with MetS-I (n=40) and MetS+I (n=33) were also selected from a previously published study on the effect of lifestyle in combination with rosiglitazone versus placebo on atherosclerosis in subjects with the metabolic syndrome [RUBENS study, (16)]. These subjects (n=73) were matched on waist and age with the subjects from the PIRAMID study (see below). For this study male subjects with increased waist circumference (≥ 94 cm) and elevated CRP levels ($\geq 1.8 \text{ mg/l}$) and two other metabolic syndrome criteria were included. Exclusion criteria for this study were type 2 diabetes (fasting blood glucose $\geq 7 \text{ mmol/l}$), manifest cardiovascular disease, use of statins, steroids or non-steroidal anti-inflammatory drugs at baseline, heart failure, QTc time interval of 450 ms or longer on baseline electrocardiogram (ECG), primary dyslipidemias, presence of potential hepatic disease (i.e. subjects with alanine aminotransferase, total bilirubin, or alkaline phosphatase levels exceeding 2.5 times the upper limit of the normal labora-

tory values), alcohol abuse (> 30 units/week) and cardiovascular magnetic resonance contraindications.

Subjects with T2DM (n=68) were selected from a recently published study on the effects of pioglitazone versus placebo on cardiovascular parameters in uncomplicated T2DM (PIRAMID) study. For detailed inclusion criteria, see (17). In short, the study included male patients with T2DM without cardiovascular disease or diabetes-related complications, BMI 25-32 kg/m², age 45-65 years and well-controlled type 2 diabetes with oral antidiabetic medication (HbA1c 6.5-8.5%). A total of n=73 subjects from the PIRAMID were matched on waist circumference and age with n=73 subjects from the RUBENS. A total of 5 subjects were excluded due to CRP levels > 15 (n=4) and missing data (n=1).

The studies were executed in accordance with the principles of the Declaration of Helsinki and were approved by the local medical ethics committee. All volunteers in these studies signed informed consent.

Analysis of CRP and ANGPTL4

CRP was determined by the Tina Quant C-reactive protein (latex) high sensitive assay according to the manufacturer's instructions (Roche, Basel, Switzerland). ANGPTL4 was measured by ELISA as detailed previously (9). Briefly, 96-well plates were coated with anti-human ANGPTL4 polyclonal goat IgG antibody (AF3485, R&D Systems) and incubated overnight at 4°C. Plates were washed extensively between each step. After blocking, 100 µl of undiluted medium or 20-fold diluted human plasma was added, followed by 2 h incubation at room temperature. A standard curve was prepared using recombinant human ANGPTL4 (3485-AN, R&D Systems) at 0.3 to 2.1 ng/well. Next, 100 µl of diluted biotinylated anti-human ANGPTL4 polyclonal goat IgG antibody (BAF3485, R&D Systems) was added for 2 h, followed by addition of streptavidin-conjugated horseradish peroxidase for 20 min, and tetramethyl benzidine substrate reagent for 6 min. The reaction was stopped by addition of 50 µl of 10% H₂SO₄, and the absorbance was measured at 450 nm.

In vitro studies

Cell culture

Human THP-1 and U937 monocytes were grown in RPMI medium containing 10% heat-inactivated FCS and 1% P/S for 10-15 passages. They were differentiated into macrophages after 2 days incubation by adding 100 ng/ml phorbol-12-myristate-13-acetate (PMA). After 2 days, PMA was removed by washing with phosphate-buffered saline (PBS) and macrophages were kept in complete medium without PMA for 2 additional days and then used for experiments. Human Peripheral blood mononuclear cells (PBMCs) were

isolated from whole blood by using BD Vacutainer Cell Preparation Tubes according to the manufacturer's instructions. Isolated PBMCs were plated in complete medium RPMI, 10% heat inactivated FCS, 1% P/S. After 2 h cells were washed and remaining attached cells were differentiated into macrophages in completed medium, containing 100 ng/ml macrophage colony stimulating factor (M-CSF), for 5 subsequent days. During differentiation medium was not refreshed.

Incubations

THP-1 cells were incubated with the TLR agonists (Sigma, Fluka, Brunwich, and InvivoGen) lipopolysaccharide (LPS, TLR4; 100 ng/ml or 1 µg/ml), S-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe, Pam2CGDPKHPKSF (Fibroblast-stimulating Lipopeptide; FSL-1, TLR2 and TLR6; 1 µg/ml), tri-acylated lipopeptide Pam3-Cys-Ser-Lys4 (Pam3, TLR1 and TLR2; 1 µg/mL), Polyinosinic-polycytidylic acid (polyI:C, TLR3; 2 µg/ml), Muramyl dipeptide (MDP, NOD2; 10 µg/ml), Flagellin (Flag, TLR5; 10 ng/ml) and/or FFA-donor intralipid (2 mM; Fresenius Kabi) for the indicated time periods at 37°C. After incubation, medium was aspirated and cells were washed. Cellular ANGPTL4 mRNA was determined by qPCR (forward primer CACAGCTGCAGACACAACCTC; reverse primer GGAGGCCAAACTGGCTTTGC) and ANGPTL4 protein in the medium was determined by ELISA as detailed above.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation or as median (interquartile range) if the assumption of normality was not met. Categorical data are presented as frequencies (%). Comparisons between continuous variables were assessed by independent t-test or one-way ANOVA, followed by Bonferroni's post hoc test for multiple comparisons, (**Table 1, Figure 1**). Comparisons between categorical data were performed with Chi-square tests (with the level of significance adjusted for the number of comparisons, **Table 1**). The correlation between clinical characteristics and ANGPTL4 was tested by Spearman's correlation coefficients (**Figure 2**) and multivariable regression analysis with logarithmically transformed ANGPTL4 values. Statistical analysis was performed using SPSS for Windows (version 17.0; SPSS, Chicago, Illinois, USA). Plots were created with GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, USA). $P < 0.05$ was considered statistically significant.

Table 1. Clinical characteristics

	Controls n=95	MetS-I n=106	MetS+I n=66	T2DM n=68
Age, years	56.5 ± 7.4	56.8 ± 5.2	57.5 ± 5.5	56.8 ± 5.3
Current smoking, n (%)	17 (18) [‡]	29 (27)	26 (39) [§]	11 (16) [‡]
Family history of CVD, n (%)	19 (20)	21 (20)	14 (21)	13 (19)
Statin use, n (%)	8 (8) [§]	8 (8) [§]	4 (6) [§]	32 (47) ^{*†‡}
Waist circumference, cm	103 ± 9	105 ± 10	107 ± 9	104 ± 10
Systolic blood pressure, mmHg	133 ± 18 ^{†‡}	146 ± 20 [§]	145 ± 18 [§]	130 ± 12 ^{†‡}
Diastolic blood pressure, mmHg	82 ± 12 ^{†‡}	88 ± 10 [§]	87 ± 11 [*]	82 ± 8 [†]
Glucose, mmol/l	5.14 ± 0.47 ^{†‡§}	5.79 ± 0.82 [§]	5.80 ± 0.86 [§]	8.44 ± 1.92 ^{*†‡}
Free fatty acids, mmol/l	0.62 (0.47-0.79)	0.58 (0.43-0.73)	0.58 (0.44-0.75)	0.58 (0.45-0.75)
Total cholesterol, mmol/l	5.63 ± 0.98 [§]	5.59 ± 1.01 [§]	5.57 ± 1.11 [§]	4.66 ± 0.93 ^{*†‡}
HDL-cholesterol, mmol/l	1.24 (1.11-1.41) ^{†‡§}	1.02 (0.89-1.22) [*]	1.02 (0.85-1.19) [*]	1.11 (0.93-1.39) [*]
Triglycerides, mmol/l	1.09 (0.84-1.38) ^{†‡§}	1.82 (1.31-2.45) [*]	1.83 (1.15-2.40) [*]	1.50 (0.90-2.20) [*]
CRP, mg/l	1.43 (0.76-2.85) ^{†§}	1.96 (0.92-2.37) ^{†§}	5.55 (4.11-8.02) ^{*†§}	3.60 (2.43-5.95) ^{*†‡}
ANGPTL4, ng/ml	3.7 (2.8-5.2) ^{†‡§}	4.6 (3.3-6.5) ^{*†§}	6.0 (3.8-7.7) ^{*†}	6.4 (5.2-8.2) ^{*†}

Values represent mean ± standard deviation or median (inter quartile range) unless indicated otherwise. Abbreviations: ANGPTL4, angiotensin-like protein 4; CRP, C-reactive protein; CVD, cardiovascular disease; HDL, high-density lipoprotein; MetS-I, metabolic syndrome without inflammation; MetS+I, metabolic syndrome with inflammation; T2DM, type 2 diabetes mellitus. Significant as compared to ^{*}healthy controls, [†]MetS-I, [‡]MetS+I, [§]T2DM (P<0.05).

RESULTS

Characteristics of the human study population

To evaluate whether low grade inflammation is associated with plasma ANGPTL4 levels in humans, we compared Controls (n=95) with subjects with MetS-I (n=106), MetS+I (n=66), and with T2DM patients (n=68).

The mean age of all male subjects combined (n=335) was 56.9±6.0 years and mean waist circumference was 105±10 cm. Clinical characteristics of the study population are described in **Table 1**. Groups matched for age and waist circumference did not differ with regard to FFA and family history of cardiovascular disease. Fasting glucose and triglyceride levels were higher, and HDL-cholesterol levels were lower in MetS-I, MetS+I and T2DM compared to Controls. Blood pressure was higher in MetS-I and MetS+I compared to Controls and T2DM. Subjects with T2DM used more statins and had lower total cholesterol compared to MetS-I, MetS+I and Controls. Furthermore subjects with MetS+I smoked more frequently than controls and subjects with T2DM. Low grade inflammation measured as CRP was highest in MetS+I [5.55 (4.11-8.02) mg/l, p<0.001 compared to all groups], followed by T2DM [3.60 (2.43-5.95) mg/l], MetS-I [1.96 (0.92-2.37) mg/l] and Controls [1.43 (0.76-2.85) mg/l].

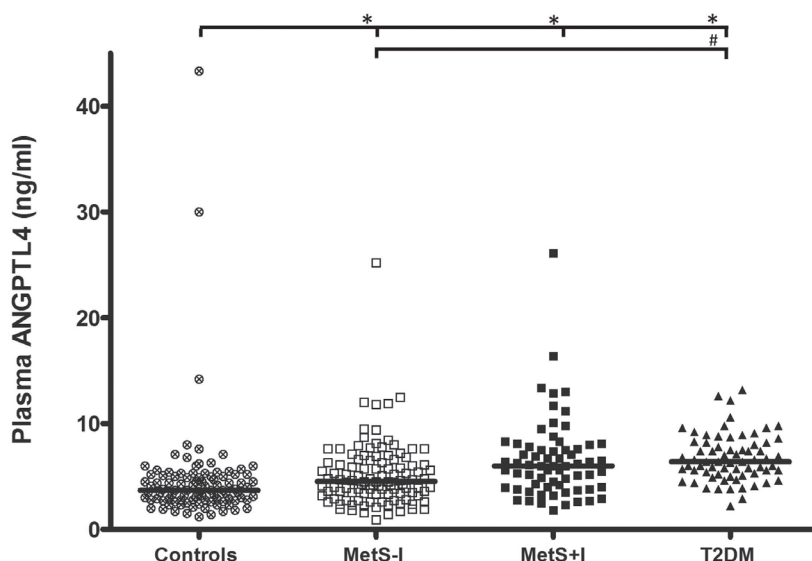


Figure 1. Plasma angiopoietin-like protein 4 (ANGPTL4) in healthy controls (Controls), patients with metabolic syndrome without (MetS-I) and with low grade inflammation (MetS+I) and patients with type 2 diabetes mellitus (T2DM). Plasma was taken from Controls, patients with MetS-I, MetS+I and T2DM, and plasma ANGPTL4 levels were determined. As compared with plasma ANGPTL4 levels [median (IQR)] in Controls [3.7 (2.8-5.2) ng/ml], ANGPTL4 levels are increased in patients with MetS-I [4.6 (3.3-6.5) ng/ml, $p=0.006$], patients with MetS+I [6.0 (3.8-7.7) ng/ml, $p<0.001$] and patients with T2DM [6.4 (5.2-8.2) ng/ml, $p<0.001$]. Furthermore, ANGPTL4 was increased in T2DM2 compared with MetS-I ($p<0.001$). Significant as compared with Controls. * $P<0.001$, # $P<0.05$.

Plasma ANGPTL4 levels

The median plasma ANGPTL4 in the total study population was 5.00 (3.50-6.80) ng/ml and ranged from 0.90 to 43.30 ng/ml. The distribution of ANGPTL4 was skewed to the right. **Figure 1** shows the plasma ANGPTL4 levels in Controls, MetS-I, MetS+I and T2DM. Plasma ANGPTL4 was higher in T2DM [6.4 (5.2-8.2) ng/ml, $p<0.001$], in MetS+I [6.0 (3.8-7.7) ng/ml, $p<0.001$] and MetS-I [4.6 (3.3-6.5) ng/ml, $p=0.006$], than in Controls [3.7 (2.8-5.2) ng/ml]. In addition, higher ANGPTL4 was found in T2DM compared to MetS-I ($p<0.001$) (**Table 1**).

Relationship between plasma Angptl4, metabolic parameters and low grade inflammation

Although groups were matched for waist circumference, in all subjects combined plasma ANGPTL4 positively correlated with waist circumference ($r=0.195$, $p<0.001$) and fasting glucose ($r=0.322$, $p<0.001$). Plasma ANGPTL4 negatively correlated with HDL-cholesterol ($r=-0.143$, $p=0.009$) and total cholesterol ($r=-0.223$, $p<0.001$). Plasma ANGPTL4 did not correlate with age ($p=0.457$), triglycerides ($p=0.055$) or FFA ($p=0.226$).

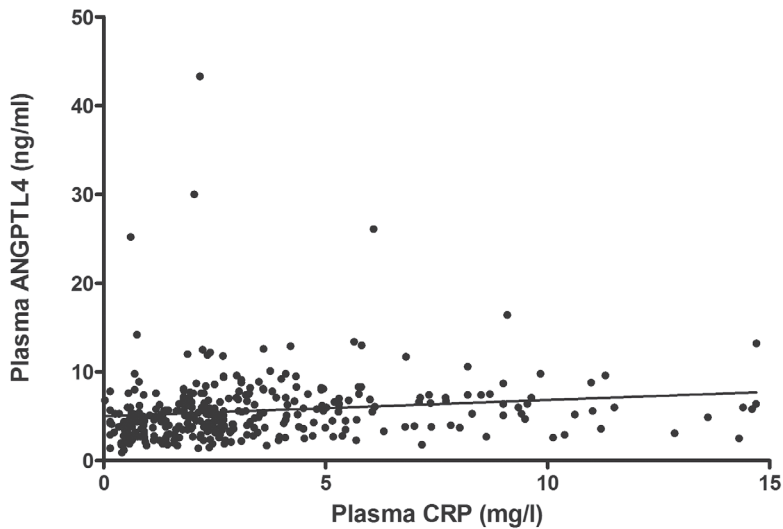


Figure 2. Correlation between plasma C reactive protein (CRP) and angiopoietin-like protein 4 (ANGPTL4). Plasma was taken from healthy controls, patients with metabolic syndrome without and with low grade inflammation and patients with type 2 diabetes mellitus, and plasma ANGPTL4 levels as well as CRP levels were determined. Plasma CRP correlates positively with plasma ANGPTL4 ($r=0.295$, $P<0.001$).

Plasma ANGPTL4 showed a positive correlation with CRP ($r=0.295$, $p<0.001$) (**Figure 2**). Multiple regression analysis revealed that the association between ANGPTL4 and CRP was independent of age, waist circumference, glucose, HDL-cholesterol, triglycerides, total cholesterol, FFA and smoking ($\beta = 0.142$, $p=0.010$).

Inflammation-dependent ANGPTL4 expression in human macrophages

Since the above described correlations do not necessarily implicate causal relationships, we performed mechanistic studies *in vitro* to get more insight into potential causality between inflammation and ANGPTL4 expression. To demonstrate that ANGPTL4 expression is sensitive to stimulation by inflammatory stimuli, we treated macrophages with various PRR agonists and determined ANGPTL4 expression (**Figure 3**). We used human THP-1 macrophages because: 1) they are human macrophages, 2) they secrete sufficient amounts of ANGPTL4 to allow quantification in the cell medium.

In human THP-1 macrophages ANGPTL4 mRNA expression (**Figure 3A**) and ANGPTL4 protein secretion (**Figure 3B**) were strongly increased by the TLR4 agonist LPS and the TLR3 agonist poly(I:C). Consistent with these data, LPS also increased ANGPTL4 mRNA expression in human U937 macrophages and human monocyte-derived macrophages (**Figure 3C**). Intralipid, a source of FFA that induces foam cell formation, also increased ANGPTL4 mRNA (**Figure 3D**) and ANGPTL4 protein (**Figure 3E**) expression in THP-1

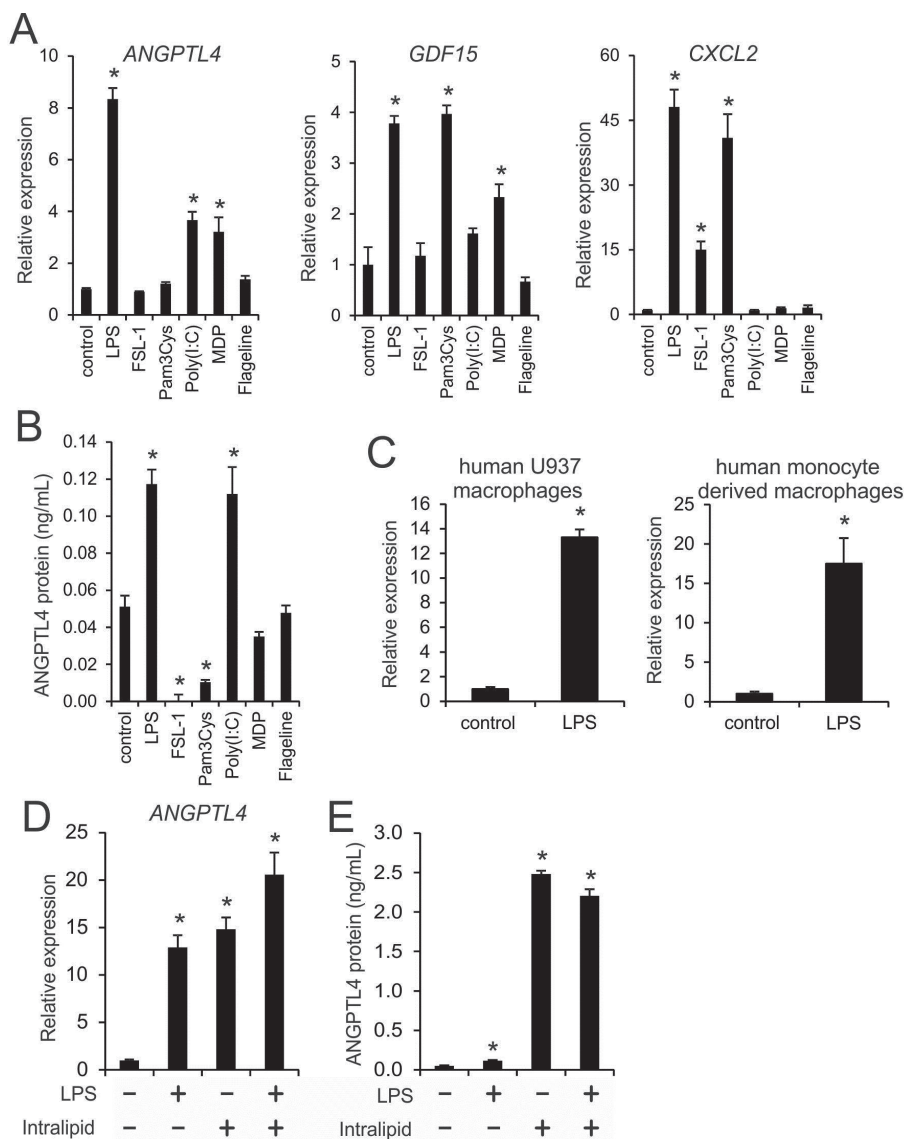


Figure 3. Inflammatory stimuli increase angiopoietin-like protein 4 (ANGPTL4) gene and protein expression in human macrophages. (A) Human THP-1 macrophages were incubated with various Toll-like receptor (TLR) agonists for 12 h, and changes in expression of ANGPTL4, Gdf15 and Cxcl2 were determined. (B) Human THP-1 macrophages were incubated with various TLR agonists for 24 h, and ANGPTL4 protein levels in medium were assessed. (C) Human U937 macrophages were incubated with LPS (100 ng/ml; 4 h) and ANGPTL4 mRNA expression was determined; human monocyte-derived macrophages were incubated with LPS (1 µg/ml; 24 h) and ANGPTL4 mRNA expression was determined. (D, E) THP-1 macrophages were incubated with LPS, intralipid, or both for 24 h and ANGPTL4 mRNA expression (D) or protein secretion (E) were determined. Values are means ± SEM (n=3-6). Differences as compared to controls were evaluated by Student's t-test. *P<0.05.

macrophages, but no clear synergy was observed between Intralipid and LPS towards induction of ANGPTL4. Taken together, these data show that inflammatory stimuli causing macrophage activation and macrophage foam cell formation markedly increase ANGPTL4 expression in human macrophages.

DISCUSSION

The main findings of our cross-sectional human study are: 1) patients with MetS and T2DM show increased plasma ANGPTL4 levels compared to Controls, 2) MetS patients with low grade inflammation show higher levels of ANGPTL4 compared to MetS patients without inflammation, 3) plasma CRP, as a marker of low grade inflammation, positively correlates with plasma ANGPTL4, independent of age, waist circumference, glucose and lipid variables.

Thus far, the expression of ANGPTL4 in numerous tissues including adipose tissue and liver was thought to be mainly regulated by PPARs. Consistent with a role of PPARs, fatty acids increase ANGPTL4 expression *in vitro* in numerous cell types (1,6-8). Furthermore, *in vivo* modulation of plasma FFA levels also modulates plasma ANGPTL4 levels (9,10), with a positive correlation observed between the change in FFA levels and change in ANGPTL4 levels (10). In our previous study, we postulated that patients with T2DM may have higher ANGPTL4 levels than healthy subjects due to elevated plasma FFA (10). Although in the present paper we do observe increased plasma ANGPTL4 levels in T2DM patients compared to controls, plasma FFA levels were not different between both groups. In addition, plasma ANGPTL4 levels were elevated in subjects with MetS compared to controls, whereas their plasma FFA levels did not differ from the healthy controls. In fact, in this cross-sectional study we were unable to detect a correlation between plasma FFA levels and plasma ANGPTL4 levels, which is likely related to the matching on visceral obesity and the subsequent limited variation in FFA between groups.

Therefore, we hypothesized that additional factors such as inflammation may contribute to the increased ANGPTL4 levels in subjects with MetS and T2DM. Indeed, we did observe a progressive increase in the plasma ANGPTL4 in males according to inflammatory status (healthy controls < subjects with MetS without inflammation < subjects with MetS with low grade inflammation and subjects with T2DM). Furthermore, in all groups combined, plasma ANGPTL4 showed a positive correlation with CRP, an established marker for low grade inflammation.

To get more insight into the causal relationship between inflammation and ANGPTL4 expression we treated human macrophages with various PRR ligands. Macrophages were used because of their sensitivity to inflammatory stimuli, not because they are suspected to be solely responsible for the changes in plasma ANGPTL4 levels observed *in vivo*. Of

all ligands tested, agonists for TLR3 (*i.e.* poly(I:C)) and TLR4 (*i.e.* LPS) consistently induced the expression and release of ANGPTL4 in different types of human macrophages. It remains unclear which intracellular pathway activated by TLR activation is responsible for induction of ANGPTL4. Also, it is unsure whether FFA, known to activate PRRs such as TLR4 in adipocytes and macrophages (11,12), may increase ANGPTL4 expression via TLR4 activation. It should be noted that the similarity in plasma FFA levels between the various groups despite major differences in plasma ANGPTL4 levels, suggests that plasma FFA are not a major determinant of ANGPTL4 in the population studied. Since CRP itself is a marker rather than a mediator of inflammation (18), the nature of the inflammatory stimuli that cause an increase in plasma ANGPTL4 in humans evidently still has to be identified. Also, it would be of interest to study the response of ANGPTL4 to inflammatory stimuli in other cell types, including human adipocytes.

We hypothesized that the inflammation-induced increase in plasma ANGPTL4 in subjects with MetS and T2DM may induce further dyslipidemia. Indeed, taken all groups together, we observed a modest negative correlation between ANGPTL4 and plasma HDL-cholesterol ($r=-0.143$; $p<0.009$). Some limitations do apply to our study. First of all, we previously showed that the ANGPTL4 ELISA measures full length ANGPTL4 and the C-terminal truncated fragment of ANGPTL4 but not the N-terminal truncated fragment. Only full length and the N-terminal fragment of ANGPTL4 influence plasma lipids. It is unclear to what extent the plasma concentrations of C-terminal and full-length ANGPTL4 reflect the concentration of N-terminal ANGPTL4. The mechanism underlying the relationship between inflammation and plasma ANGPTL4 levels is unknown as yet, and probably involves causal stimuli other than CRP that merely represents a marker of inflammation. Further studies into the causal relationships are thus required. Also, it is unclear why CRP is lower in T2DM compared to subjects with MetS with low grade inflammation. It should be noted that well-controlled diabetic patients with an HbA1c between 6.5-8.5%, a BMI between 25-32 kg/m², blood pressure < 150/85 mmHg and no history of diabetes related complications were included, who were treated with drugs such as statins with pleiotropic anti-inflammatory effects. Furthermore, higher CRP in subjects with MetS with low grade inflammation may be explained by a higher percentage of smokers in that group. Also, from the current study set up it is unclear whether the relationship between ANGPTL4 and CRP in humans is solely explained by activation of macrophages. Other cell types and tissues may also be involved.

In conclusion, we provide a novel link between inflammation and ANGPTL4. An increased inflammatory state in subjects with MetS and T2DM was associated with higher plasma ANGPTL4 levels. Additionally, a positive correlation was observed between plasma CRP and ANGPTL4. Although *in vitro* studies confirmed that PRR ligands increase ANGPTL4 expression, further studies into the precise mechanisms underlying the relationship between inflammation and ANGPTL4 in humans are warranted.

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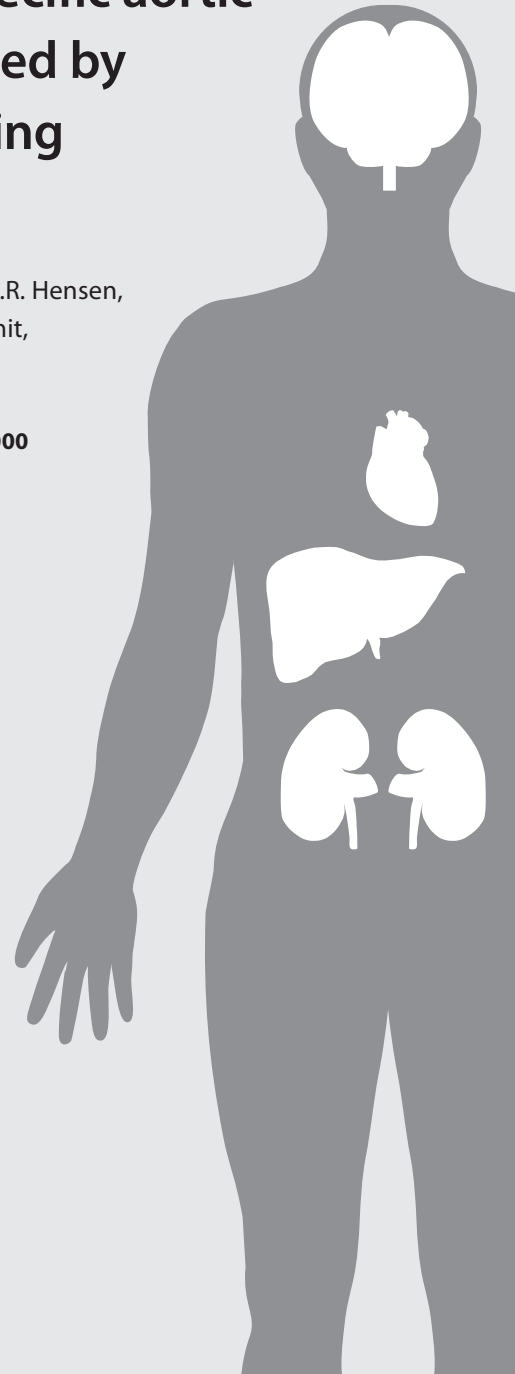
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Chapter 4

Short-term effects of a standardized glucose load on region-specific aortic pulse wave velocity assessed by magnetic resonance imaging

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ABSTRACT

Purpose

To assess the short-term effects of a standardized oral glucose load on regional aortic pulse wave velocity (PWV) using two-directional in-plane velocity encoded magnetic resonance imaging (MRI).

Materials and Methods

A randomized, controlled intervention was performed in 16 male subjects (mean±standard deviation: age: 59±7 years, body mass index: 28±3 kg/m²) with impaired fasting glucose. The intervention consisted of an oral glucose load (75 grams of carbohydrates in 300 ml water) at one study day and water (300 ml) at the other study day. PWV was measured using multislice two-directional in-plane velocity-encoded MRI.

Results

PWV in the proximal aorta at 1 hour post-glucose load decreased compared to PWV 1 hour post-water (delta PWV: -1.0 ± 2.6 vs. 0.6 ± 2.0 m/s, $P=0.02$). Eight responding subjects showed a significant decrease in PWV of the proximal aorta after the glucose load and revealed a lower blood pressure and were significant less obese ($P=0.037$) compared to non-responders. There was no significant change in PWV of the distal aorta at 1 hour post-load comparing both intervention groups.

Conclusion

A standardized oral glucose load induces a decrease of the proximal, but not of the distal, aortic PWV. Regional response of aortic PWV may be associated with features of the metabolic syndrome.

INTRODUCTION

Insulin plays an important role in hemodynamic regulation by modulating vascular tone. Nitric oxide (NO) release from the endothelium may be implicated in this process and induces vasodilation (1). The effect of insulin on vascular tone may be region-specific for various vascular beds and locations. In vitro the contribution of NO to endothelium dependent vasodilation is significantly larger in central arteries than in distal microvessels (2). Furthermore, Westerbacke et al. demonstrated a decrease in large peripheral arterial stiffness in healthy subjects after infusion of physiologic insulin concentrations (3). They assessed vascular response from a change in augmentation index, which was assessed by applanation tonometry of the radial artery.

Aortic pulse wave velocity (PWV) is a surrogate marker for central arterial stiffness and a prognostic marker for cardiovascular disease (4). PWV is most frequently measured by tonometry and echo Doppler. In contrast to these traditional methods, magnetic resonance imaging (MRI) in combination with velocity-encoding enables regional assessment of the PWV in multiple locations in the aorta due to its accuracy in determining the true path length of the propagating wave in the aorta. Moreover, MRI-based estimates of regional aortic PWV have been well validated against intra-arterial pressure measurements as the gold standard (5).

Our hypothesis is that a glucose load may induce local changes in aortic stiffness mediated by increases in plasma insulin and possibly NO release. Accordingly, the purpose of our study was to assess the short-term effects of a standardized glucose load on regional aortic PWV using the two-directional in-plane velocity encoded MRI technique.

MATERIALS AND METHODS

Subjects

Sixteen men (mean $\pm$ SD: age: 59 $\pm$ 7 years, BMI: 28 $\pm$ 3 kg/m²) for the current study were recruited from general health care practices. All volunteers participated in cardiovascular screening and fulfilled the criteria for impaired fasting glucose [IFG; fasting glucose $\geq$ 5.6 mmol/L and <7 mmol/L(6)]. The screening visit consisted of a medical history, physical examination and laboratory screening in the fasted state (including plasma glucose, lipids, creatinine, alanine aminotransferase, aspartate aminotransferase). Inclusion criteria were: males aged between 18 and 75 years. Exclusion criteria were: previously diagnosed diabetes mellitus, any chronic disease, history of cardiovascular disease, use of any of the following medication: antihypertensives, glucose lowering medication, statins or fibrates, blood pressure >160/100 mmHg [grade 2 or 3 hypertension according to guidelines of European Society of Hypertension and European Society of Cardiology

(7)], smoking or any contra-indication for MRI. Hypertension was defined as systolic and/or diastolic blood pressure of greater than or equal to 140 mmHg and/or greater than 90 mmHg.

This study was executed in accordance with the principles of the Declaration of Helsinki, it was approved by the local medical ethics committee and after the nature of procedures had been explained all participants signed informed consent.

Study design

This randomized, controlled intervention study consisted of two study days, with at least one week between the study days. The intervention consisted of a single oral drink with 300 ml water with 75 grams of carbohydrates (Dextropur, Dextro Energy GmbH&Co. KG, Germany) on one day and the other study day the intervention consisted of 300 ml water only. The sequence of both interventions was randomly assigned. Participants were instructed to refrain from alcohol and caffeine and to avoid exercise during 24 hours before both study days. Subjects consumed the evening before both study days the same standard meal, consisting of on average 480 kcal (37 energy percent (En%) carbohydrates, 44 En% fat). After a twelve hours overnight fast, participants were admitted at the research unit of the university hospital. After measurement of weight and blood pressure, an intravenous cannula was inserted and a first blood sample was taken. Subsequently, participants were transferred to the MRI scanner for the first MRI scan (see below for scan protocol) in a temperature-controlled room. After the first MRI scan subjects received the glucose or control drink. Blood samples were drawn 30, 60, 90 and 120 minutes after ingestion of the drink. PWV measurements were repeated at 60 and 120 minutes after the ingestion of the drink.

Blood sampling and analysis

Blood samples were collected in serum-gel and potassium-EDTA tubes (Sarstedt Monovette®, Sarstedt, Germany). The EDTA tubes were immediately put on ice after sampling. All samples were centrifuged (3540g at 4°C, during 20 minutes) and stored in aliquots at -80 °C until analysis. Samples were measured in one assay run. Plasma glucose was measured on a Modular P800 chemistry analyzer of Roche Diagnostics (Mannheim, Germany, total CV: 1.4%). Serum insulin was measured using an Immulite 2500 analyzer of Siemens Diagnostics (Breda, The Netherlands, total CV 4.7%). The Homeostasis Model Assessment-Insulin Resistance Model (HOMA2 calculator 2.2.2 2007; www.dtu.ox.ac.uk) was used to calculate insulin resistance.

Pulse wave velocity

Participants were studied in supine position using a 1.5 Tesla whole-body MR scanner (Gyroscan ACS/ NT15, Philips, Best, The Netherlands). The body coil and a five element

cardiac receiver coil were used for data acquisition. PWV was determined using multislice, two-directional in-plane velocity-encoded (VE) MRI. This technique has recently been described in detail and will be summarized here (5). PWV is calculated by the ratio of the distance along the aortic center line (Δx) and the transit-time of the propagating systolic pulse wave between two measurement sites (Δt). A volume slab consisting of three slices was acquired to image the ascending aorta, aortic arch and the descending aorta in double-oblique sagittal orientation, using a steady-state free-precession sequence. Scan parameters included: field-of-view (FOV): $450 \times 270 \text{ mm}^2$, slice thickness of 10 mm, echo time (TE) of 1.4 ms, repetition time (TR) of 2.9 ms, flip angle of 50 degrees. Bidirectional in-plane VE MRI was acquired in anterior-posterior (AP) and feet-head direction (FH) with free breathing and retrospective ECG-gating using the orientation of the 3-slice volume slab. Scan parameters included: velocity sensitivity of 150 cm/s, TE of 2.4 ms, TR of 4.3 ms, flip angle of 10 degrees, number of signal averages of 2.

In the three-slice volume slab, the aorta was manually segmented, from the aortic root to the descending aorta at the level of the 2nd lumbar vertebrae. Segmentations were performed by one observer (with 3 years of experience in cardiac MRI) supervised by a senior researcher (with 15 years of experience in cardiac MRI). Image analysis was performed using in-house developed MASS software (Leiden University Medical Center, Leiden, the Netherlands). Perpendicular to the automatically defined center line of the aorta, 200 sample chords were equidistantly distributed. From each sample chord, the maximal aortic flow velocity in the direction parallel to the centerline was calculated for each cardiac phase, resulting in 200 time-velocity curves (schematic represented in **Figure 1**). From all 200 time-velocity curves, the time-to-half-peak was automatically determined, using difference between maximum and minimum aortic flow velocity along the systolic upslope. The length of the aorta was manually determined by placing a poly-line along the center line of the aorta in the three-slice volume slab. Regional PWV was determined from the inverse slope, calculated by linear regression between the positions along the centerline versus the transit-time relation.

We calculated the PWV proximal aorta (from ascending aorta to proximal descending aorta, both defined in a plane transecting the aorta at the level of the pulmonary trunk), distal aorta (below proximal aorta to the level of the 2nd lumbar vertebrae) and the total PWV (proximal aorta and distal aorta summed). A change in PWV relative to baseline measurement was considered significant if change in PWV exceeded 2x coefficient of variance (COV) in PWV-assessment. The COV of this method was determined previously from repeated in-plane PWV-measurements (5) and was 10% for aortic PWV of the proximal and 12% for aortic PWV of the distal aorta.

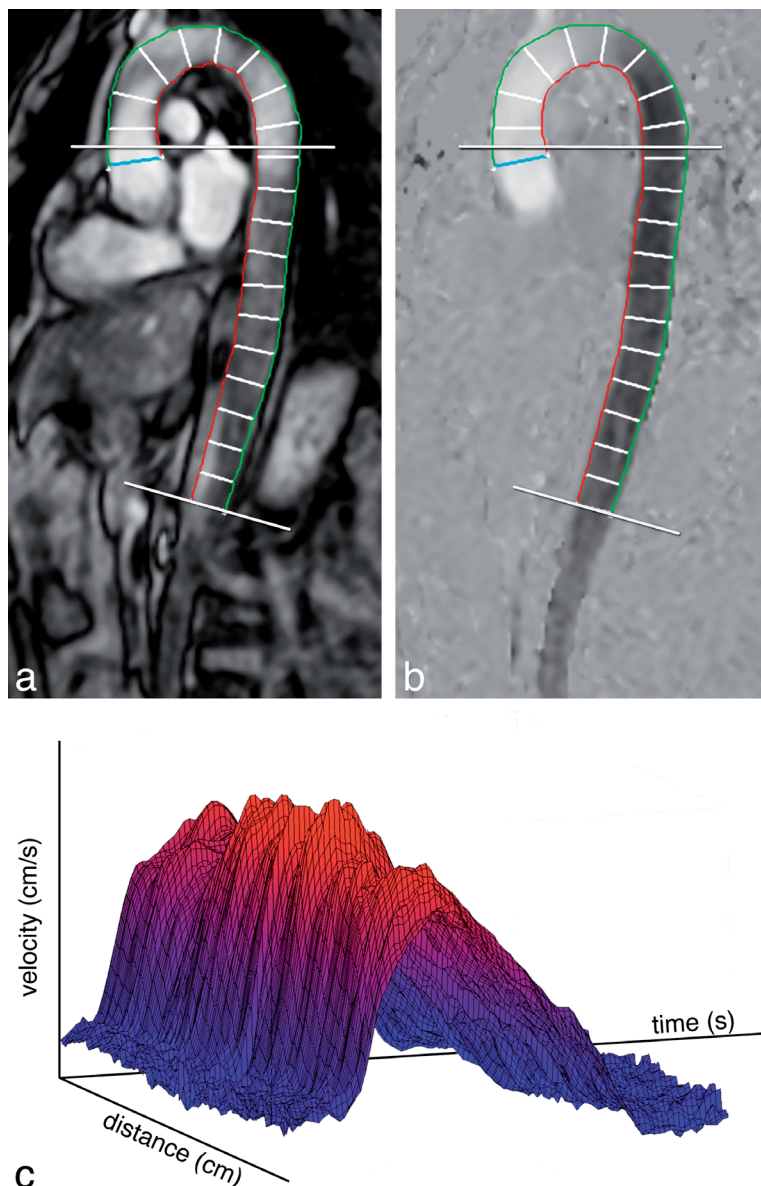


Figure 1. Pulse wave velocity (PWV) assessment by multislice two-directional inplane velocity-encoded Magnetic Resonance Imaging (MRI). The aorta was manually segmented along the ascending aorta from the level of the pulmonary trunk to the descending aorta at the level of the second lumbar vertebrae (A) and contours were copied to the velocity images (B represents feet-head encoded velocity). The 200 sample chords were equidistantly distributed along the centerline and for each chord, the maximal aortic flow velocity along the centerline of the aorta was determined for each cardiac phase, resulting in 200 time-velocity curves (C). The arrival times of each of the 200 wave forms are plotted against the position along the centerline and PWV is determined from the inverse slope by linear regression between arrival time and position.

Statistical analyses

Data are expressed as mean $\pm$ standard deviation (SD). Comparisons of the change in PWV between the two interventions were performed with a paired samples t-test for continuous variables and a Chi-Square test was used to compare the number of subjects with a change in aortic PWV. A p-value <0.05 was considered statistically significant. Statistical analyses were performed using SPSS (SPSS Inc. Chicago, Illinois, USA). Graphs were created using GraphPad Prism 5.01 (GraphPad Software, Inc., La Jolla, USA).

RESULTS

Sixteen men were included (age: 59 ± 7 years, body mass index: 28 ± 3 kg/m²). Baseline characteristics of the study population are presented in **Table 1**.

Glucose and insulin concentrations significantly increased over time after the glucose load (**Figure 2A&B**, $P < 0.05$) compared to the water load.

Aortic PWV was not different between both study days at baseline (aortic PWV before the glucose load vs. water: proximal aorta 8.0 ± 3.0 vs. 7.0 ± 2.2 m/s, $P > 0.05$; distal aorta 9.8 ± 4.7 vs. 9.2 ± 3.1 m/s, $P > 0.05$ and total aorta 8.8 ± 1.9 vs. 8.2 ± 1.7 m/s, $P > 0.05$).

The change (delta) in aortic PWV at one hour after the intervention was calculated as aortic PWV at 1 hour minus aortic PWV at baseline. Delta aortic PWV at two hours was calculated in a similar way. Delta PWV in the proximal aorta at one hour after the glucose load was significantly lower compared to the delta PWV one hour after the control load

Table 1. Characteristics of the study population at screening

N	16
Age (years)	59 ± 7
Waist circumference (cm)	101 ± 9
Body mass index (kg/m ²)	28.0 ± 2.9
Serum glucose at screening (mmol/l)	5.6 ± 0.4
HbA1c (%)	5.4 ± 0.3
Plasma cholesterol (mmol/L)	6.6 ± 1.0
HDL cholesterol (mmol/L)	1.5 ± 0.3
Cholesterol/ HDL ratio	4.7 ± 1.0
Plasma triglycerides (mmol/L)	1.9 ± 1.3
Heart rate (beats/min)	63 ± 10
Systolic blood pressure (mmHg)	136 ± 11
Diastolic blood pressure (mmHg)	91 ± 6
Hypertension, n (%)	11 (69)

Values are means $\pm$ standard deviation unless otherwise stated. HDL = high density lipoprotein. Hypertension = blood pressure $\geq 140/90$ mmHg

(delta PWV proximal aorta 1 hour after the glucose load -1.0 ± 2.6 vs. water 0.6 ± 2.0 m/s, $P=0.02$). The delta PWV of the proximal aorta was not significantly different at two hours after the glucose or control load.

The glucose load did not affect the change in aortic PWV of the distal and the total aorta at respectively 1 and 2 hours. (**Table 2** delta PWV distal aorta one hour after the glucose load: -0.4 ± 1.9 vs. water -0.1 ± 1.7 m/s $P>0.05$; resp. after two hours glucose load -0.7 ± 2.1 vs. water 0.1 ± 2.3 m/s $P>0.05$; delta PWV total aorta: one hour after the glucose load -0.8 ± 1.7 vs. water -0.3 ± 1.5 m/s $P>0.05$ resp. after two hours glucose load 0.2 ± 1.8 vs. water 0.0 ± 1.0 m/s $P>0.05$).

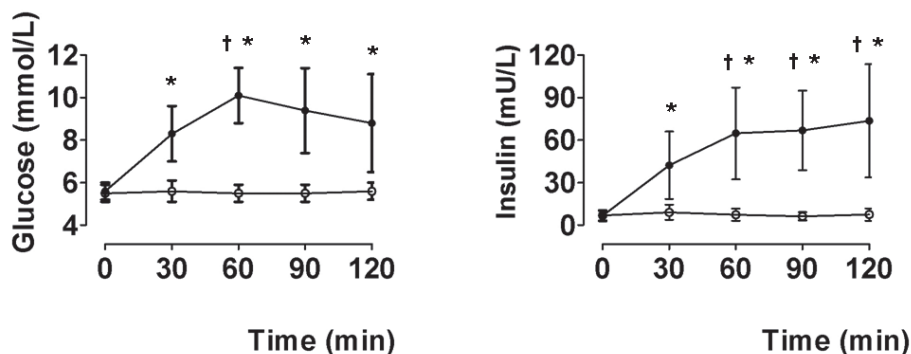


Figure 2. Plasma glucose and insulin concentrations during 120 min after a standardized oral glucose and water load. Plasma glucose concentrations (left panel) and plasma insulin concentrations (right panel) during 120 min after a 75 gram glucose load with 300 mL water (closed circles with continuous line) versus 300 mL water only (water, open circles with dotted line). The * $P<0.05$ compared with baseline in general linear model for repeated measures with Bonferroni post hoc test, † $P<0.05$ compared with 30 min in general linear model for repeated measures with Bonferroni post hoc test.

Table 2. Delta aortic PWV one and two hours after the glucose load vs. water

	Mean (sd) glucose load N=16	Mean (sd) Water N=16	P between groups
Delta PWV proximal aorta m/s			
1 hour	-1.0 ± 2.6	$+0.6 \pm 2.0$	0.02
2 hours	$+0.2 \pm 2.6$	-0.8 ± 1.8	0.13
Delta PWV distal aorta m/s			
1 hour	-0.4 ± 1.9	-0.1 ± 1.7	0.7
2 hours	-0.7 ± 2.1	$+0.1 \pm 2.3$	0.5
Delta PWV total aorta m/s			
1 hour	-0.8 ± 1.7	$+0.2 \pm 1.8$	0.5
2 hours	-0.3 ± 1.5	$+0.003 \pm 0.98$	0.8

Values are means $\pm$ standard deviation. PWV = pulse wave velocity

A significant change in proximal aortic PWV defined as a decrease in PWV exceeding $2\times\text{COV}$ was observed in eight subjects one hour after the glucose load, whereas a decrease was found in one subject under the control conditions ($P<0.05$, **Figure 3**). For the distal aorta one hour after the intervention there was no significant difference in the number of subjects with a decrease in aortic PWV comparing the glucose vs. the water load (glucose $n=3$ vs. water $n=1$, $P>0.05$, **Figure 3**). Subjects who showed a response by a decrease in proximal aortic PWV at one hour after the glucose load were significantly less obese as defined by waist circumference (98 ± 6 cm) compared to subjects with no change in proximal aortic PWV (104 ± 3 cm, $p=0.037$). Blood pressure was lower (133 ± 10 vs. 140 ± 13 mmHg, $P>0.05$) but like other baseline characteristics did not differ significantly between groups. Furthermore there were no differences in the number of subjects with an increase or decrease in aortic PWV at two hours comparing the two interventions.

Changes in PWV did not correlate with measures of insulin resistance including delta glucose, delta insulin, area under the curve and HOMA-IR.

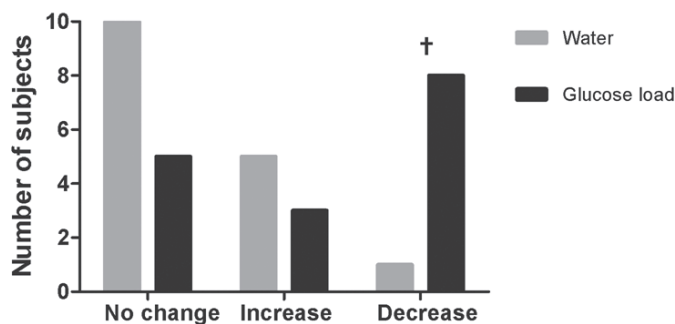


Figure 3. Change in pulse wave velocity (PWV) in proximal aorta after a standardized oral glucose or water load. Number of subjects with no change, decrease or increase in PWV assessed in the proximal aorta during first hour after a 75-g glucose load with 300 mL (black bar) versus 300 mL water only (gray bar). † $P<0.05$ between groups

DISCUSSION

The aim of the current study was to investigate the short-term effect of a standardized glucose load on region-specific aortic pulse wave velocity measured by two-directional in-plane velocity encoded MRI. The main finding of our study is that a standardized oral glucose load decreases aortic PWV significantly within one hour compared to water. Secondly this decrease in MR assessed aortic stiffness was region-specific, because it was observed only in the proximal, but not in the distal aorta. Finally, the subjects who showed a response in proximal aortic stiffness were less obese and had lower blood

pressure than the non-responders, possibly indicating a link between response and features of the metabolic syndrome.

Our results (i.e. a decline in arterial stiffness induced by an elevated glucose concentration) are in line with Westerbacka et al. (3) who demonstrated a decrease in central arterial stiffness in nine healthy males within one hour after infusion of insulin during a euglycemic clamp study. However, vascular response was assessed by augmentation index with the use of applanation tonometry recorded from the periphery (radial artery). The current study is the first to demonstrate the effect of a glucose load on regional aortic stiffness assessed by MRI measured PWV.

Interestingly, Kawano et al. (8) observed a decrease in endothelium-dependent vasodilation of the brachial artery in response to a standardized glucose load, measured as flow mediated vasodilation after sublingual nitroglycerin. Furthermore, Lambert et al. (9) observed no acute effects on carotid compliance of combined hyperglycemia and hyperinsulinemia on carotid compliance. Apparently, different measurement techniques and the measurement at different peripheral locations may yield dissimilar results.

Furthermore, most studies have not controlled for recent food intake, although recommendations suggest that subjects refrain from smoking, eating and drinking beverages containing caffeine before measurement of arterial stiffness. The measurements in our study were performed in controlled postabsorptive conditions with standardized intake and physical activity 24h before assessing PWV. Furthermore we used a well-characterized, non-smoking population without medication use and co-morbidities. Male volunteers were chosen to exclude the confounding effect of changes in reproductive hormone level. Each subject served as his own control, which enabled paired statistical analysis, which is able to detect small intra-individual effects.

The observed decrease in arterial stiffness was region-specific: it was observed in the ascending aorta and aortic arch, but not in the distal aorta. Various vascular beds and locations may react differently to the effect of insulin and other vasoactive influences. In vitro the contribution of NO dependent vasodilation is larger in central arteries than in distal microvessels (2). This may be explained by a smaller contribution of endothelium derived hyperpolarizing factor (i.e. molecules involved in the regulation of vascular tone, defined as the response that persists in the presence of combined inhibition of NO and prostacyclin) in larger arteries which was shown in an experimental study in human arteries (10). Consistent with variation in vascular reactivity to mediators of vasodilation, regional variation in the elastin versus collagen ratio exists within the aorta, with the highest ratio in the proximal segment of the aorta compared to the distal segment (11-13). Therefore, it is possible that the effect of postprandial hyperinsulinemia would have less impact on the distal segment. As the distal segment contains more collagen it is more affected by processes like aging and cross-linking by advanced glycation end products and this segment may be resistant to the vasodilatory effect of insulin.

The lack of response in some male volunteers may be related to their higher blood pressure and significant larger waist circumference. Impaired NO synthesis has been reported in conditions like the metabolic syndrome which diagnosis is based on the presence of conditions including central obesity and hypertension (14). In addition an impaired insulin-induced decrease in arterial stiffness was shown in human obesity (15).

This observation was somewhat unexpected in this rather small series of subjects, but provides the basis for further hypothesizing that there may a link between regional vascular response and insulin-resistance which is a key component of obesity-related disorders like the metabolic syndrome. Further studies are required to evaluate this possible mechanism.

Our study had several limitations. First arterial stiffness was measured in men who fulfilled the criteria for impaired fasting glucose. These volunteers were accessible for research as this group participated in cardiovascular risk screening. Although this middle-aged group did not have a history of cardiovascular disease, nor used blood pressure or lipid lowering medication, more than 50% of the volunteers were diagnosed with hypertension and they were at risk for diabetes. Therefore, the findings of this study cannot be generalized to healthy men as this may lead to an underestimation of the physiological effect of a glucose load on aortic stiffness. Finally, a post glucose load decline in PWV may potentially have occurred at different time points than those that were chosen for testing. However, the time required for MRI measurements imposed a practical limitation on the number of subsequent tests that could be performed after the glucose load.

Arterial PWV, especially of the aorta, has emerged as an important independent predictor of cardiovascular mortality (4,16). Aortic stiffness will increase the workload of the heart resulting in a higher risk of left ventricular hypertrophy and cardiac failure. Aortic arch PWV is associated with cardiac and cerebral damage (17). Furthermore stiffening of the aorta leads to higher pulsatile pressure traveling to microvascular beds in the kidneys and brain (18). Regional abnormalities in the vascular actions of insulin may be of relevance in the pathogenesis of macro- and microvascular disease in insulin resistant states like the metabolic syndrome and type 2 diabetes. Further research is needed to investigate determinants of the aortic PWV in both healthy and insulin resistant subjects.

In conclusion, a standardized oral glucose load elicits an acute transient decrease of the PWV in the proximal, but not in the distal aorta and this regional response may be associated with features of the metabolic syndrome. Therefore these results indicate that two-directional in-plane velocity-encoded MRI enables evaluation of region-specific changes of PWV in response to a glucose load.

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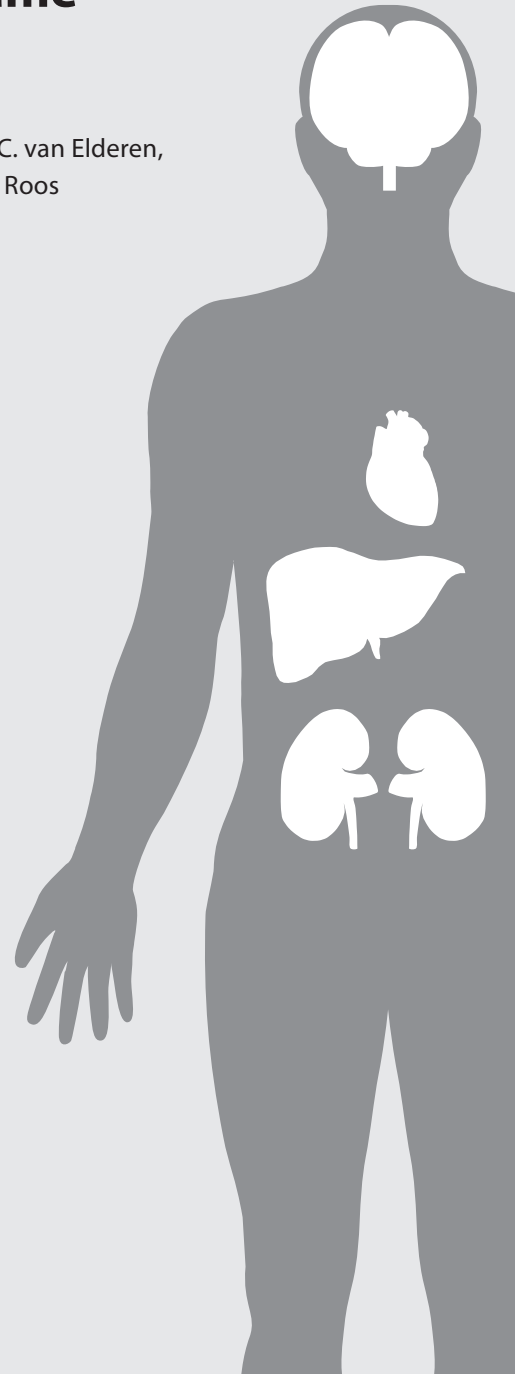
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Chapter 5

Impact of type 1 diabetes mellitus on regional grey matter volume

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Jeroen van der Grond, Johannes W.A. Smit, Albert de Roos

Submitted



ABSTRACT

Background

Type 1 diabetes mellitus (T1DM) is associated with brain damage, such as white matter hyperintensities and early development of cerebral atrophy, and impaired cognitive functioning. Metabolic factors, like hyperglycemia, and vascular complications have been proposed to affect the brain in T1DM. Cortical grey matter and the thalamus and hippocampus, two subcortical structures, are affected in patients with T1DM. No studies have yet investigated the effects on other subcortical grey matter structures. Our objective was to assess, with the advanced magnetic resonance (MR) imaging technique voxel-based morphometry (VBM), whether volumes of the basal ganglia differ between patients with longstanding T1DM and healthy controls.

Methods

Sixty-two patients with T1DM and 62 age and gender matched healthy controls underwent MR imaging of the brain. Volumes of the basal ganglia, amygdala, hippocampus and thalamus were assessed with VBM.

Results

T1DM patients showed volume loss of all of these grey matter areas, except for the amygdala. Furthermore, after correcting for potential confounding factors, the thalamus, hippocampus and putamen were still significantly smaller.

Conclusions

Using MR VBM technique, volume loss of the basal ganglia, hippocampus and thalamus is observed in patients with type 1 diabetes compared to controls.

INTRODUCTION

Type 1 diabetes mellitus (T1DM) is associated with micro- and macrovascular complications (1,2). It has become increasingly clear that T1DM also affects the brain (3,4). Several structural brain abnormalities such as white matter hyperintensities, brain infarctions and the early development of cerebral atrophy (5-7) have been observed by magnetic resonance imaging (MRI). Cross sectional studies have demonstrated that generalized white and grey matter volume loss in patients with T1DM is associated with cognitive functioning and even dementia (8). Both hyperglycemia and vascular complications have been proposed as underlying mechanisms in T1DM associated declined cognitive functioning and volume loss, but the pathogenesis is complex and incompletely understood (7,9-11).

Although previous studies have shown that T1DM is associated with grey matter density changes in general (7) and focal cortical regions and the left thalamus (12,13) and hippocampus (14,15), there are no data on the volumes of subcortical structures including the basal ganglia and amygdala in T1DM. Since the thalamus and the hippocampus are subcortical structures, we hypothesized that other subcortical structures, like the basal ganglia and the amygdala could also be affected in patients with T1DM.

Voxel-based morphometry (VBM) is a relatively novel and sophisticated analysis technique which enables to test for regional differences in brain volume. VBM enables to identify subtle grey matter alterations (16). As regional specific reductions in grey matter density in areas responsible for language processing and memory have been found in T1DM (12) we set out to perform in depth grey matter studies in T1DM, using VBM. We hypothesized that subcortical structures differ between patients with longstanding T1DM and healthy controls.

Methods

Study participants

Patients with T1DM were recruited from the local outpatient clinic between February 2008 and January 2010. For inclusion, patients had to be older than 18 years and diagnosed with T1DM. A total of 62 patients (34 men, 28 women; mean age 51.6 ± 12.4 years) gave written informed consent to participate in the study. Healthy volunteers were recruited by advertisement in local newspaper. Sixty-two age and gender matched controls were enrolled in the study. Exclusion criteria for all participants were congenital aortic/heart disease, evidence of aortic valve stenosis or insufficiency, a history of stroke and general contraindications to MRI, like claustrophobia or a pacemaker. T1DM was defined according to WHO criteria (17).

Medical data were obtained in all participants by standardized interviews and physical and laboratory examinations. Duration of T1DM in years was estimated as the time passed between the reported age of diagnosis and the MRI examination. Blood pressure and heart rate were measured using a semiautomated sphygmomanometer (Dinamap, Critikon, Tampa, Fla, USA).

Hypertension was defined as: systolic blood pressure > 140 mmHg and/or diastolic blood pressure > 90 mmHg, on repeated physical examination before antihypertensive therapy was instituted and according to the criteria of the European Society of Hypertension (18), or a blood pressure > 140/90 mmHg at time of MR imaging. Furthermore, smoking status (i.e. non-smoker or current smoker), body mass index (BMI) and high-density lipoprotein (HDL), total cholesterol, triglycerides, creatinine, glycated hemoglobin (HbA1C) in T1DM and fasting glucose in controls were determined. Renal function defined as estimated glomerular filtration (GFR) rate was calculated with the Modification of Diet in Renal Disease (MDRD) equation: $186 * (\text{serum creatinine } \mu\text{mol/L} / 88,4)^{-1.154} \times (\text{age})^{-0.203}$ ($\times 0,742$ if female)). Retinopathy was diagnosed based on fundoscopy.

This study was approved by the local medical ethics committee and was conducted according to the principles in the Declaration of Helsinki.

MRI protocol

All brain MRIs were performed on a 3.0 Tesla (Achieva; Philips Medical Systems, Best, The Netherlands). Brain MRI consisted of a 3 dimensional T1 (3D T1) sequence for brain volume assessment. 3D T1 acquisition parameters were: repetition time (TR) 9.8 ms, echo time (TE) 4.6 ms, flip angle (FA) 8°, field of view (FOV) 224 mm, 192x152 acquisition matrix, 256x256 reconstruction matrix, slice thickness 1.2 mm, 120 slices, no slice gap).

MRI post processing

All MRI scans were analyzed using different tools of FSL (FMRIB Software Library) (19). Whole brain volume, grey and white matter volumes were calculated using the FSL-tool SIENAX (Structural Image Evaluation, using Normalization, of Atrophy) (20,21). SIENAX starts by extracting brain and skull images from the single whole-head input data (22). The brain image is then affine-registered to MNI152 space (19), using the skull image to determine the registration scaling. This is primarily done in order to obtain the volumetric scaling factor, to be used as a normalization for head size. Next, tissue-type segmentation with partial volume estimation is performed to calculate total volume of brain tissue, including separate estimates of volumes of grey matter and white matter (23). To determine the volume of the subcortical twin structures nucleus accumbens, amygdala, caudate nucleus, hippocampus, pallidum, putamen and thalamus FMRIB's Integrated Registration and Segmentation Tool (FIRST) was used. FIRST starts by registering all im-

ages to MNI152 templates. Secondly it fits models for all different structures (meshes) to the images and finally applies boundary correction for the volumetric output.

A visual inspection of all registrations was performed and all final segmentations were visually inspected for accuracy.

Statistical analysis

Statistical analysis was performed by using SPSS (SPSS, Chicago, Illinois, USA), version 20. To compare clinical characteristics between T1DM and age and gender matched controls paired samples t-test for continuous variables with a normal distribution and nonparametric tests for non-normally distributed variables were used. Furthermore the McNemar test for dichotomous variables was used. Continuous variables are expressed as mean $\pm$ standard deviation (sd) or if non-normally distributed as median (interquartile range) and categorical variables were presented as frequencies and percentages.

The left and right volumes of the subcortical structures including the basal ganglia, the hippocampus and thalamus were added up and divided by 2 in order to obtain mean volumes.

Multivariate linear regression analyses were performed to assess the independent contribution of T1DM to the volumes of subcortical structures, by controlling for the following confounders: age, gender, GFR, smoking, BMI, hypertension, HDL-cholesterol and triglycerides. The β -regression coefficients and p-values are reported. Significance was reached when $p < 0.05$ (two-tailed).

RESULTS

Three-dimensional T1-weighted MRI of the brain was performed in 62 patients with T1DM and 62 age and gender matched volunteers (52 ± 12 years, 55% male). Mean T1DM duration was 27.4 ± 12.7 years, all patients used insulin and diabetic retinopathy was present in 47 (76%) diabetic patients. The clinical characteristics of the study population are shown in **Table 1**. There were no differences between groups with respect to smoking, body mass index and triglycerides. Cholesterol was lower and HDL higher in patients with T1DM compared to controls ($p = 0.011$ respectively 0.006). Twenty eight (45%) T1DM patients and 1 control subject used lipid lowering medication. Patients with T1DM were more often diagnosed with hypertension (37% vs 5%, $p < 0.001$) and used more often blood pressure lowering drugs (34 % vs 2 %, $p < 0.001$). Renal function estimated by eGFR was higher in T1DM (92.3 ± 19.4 ml/min/ 1.73^2) compared to healthy volunteers (82.4 ± 12.4 ml/min/ 1.73^2 , $p < 0.001$).

Table 1. Clinical characteristics

	Type 1 diabetes mellitus (n=62)	Healthy controls (n=62)	P value
Male sex, n (%)	34 (54.8)	34 (54.8)	1.000
Age, years	52 ± 12	52 ± 13	0.381
Type 1 diabetes duration, years	27.4 ± 12.7	-	
Hypertension, n (%)	23 (37.1)	3 (4.8)	<0.001 ^a
Current smoker, n (%)	8 (12.9)	8 (12.9)	1.000
Body mass index, kg/m ²	25.3 ± 3.3	25.4 ± 3.4	0.791
ACE inhibitors, n (%)	10 (16.1)	0 (0)	0.004 ^a
AT2 antagonist, n (%)	11 (17.7)	0 (0)	0.125
Ca antagonist, n (%)	5 (8.1)	0 (0)	0.125
Diuretics, n (%)	7 (11.5)	0 (0)	0.016 ^a
B blocker, n (%)	6 (9.7)	1 (1.6)	0.125
Use of statin, n (%)	28 (45.2)	1 (1.6)	<0.001 ^a
Retinopathy, n (%)	47 (77)	-	
HbA1c, %	7.7 ± 1.1	-	-
HbA1c, mmol/mol	60.9 ± 11.7		
Fasting plasma glucose, mmol/l	-	5.22 ± 0.86	-
Creatinine, mmol/l	74.8 ± 12.4	81.6 ± 11.8	0.001 ^a
Estimated GFR (MDRD), ml/min	92.3 ± 19.4	82.4 ± 12.4	<0.001 ^a
Total cholesterol, mmol/l	4.85 ± 0.95	5.33 ± 1.30	0.011 ^a
HDL cholesterol, mmol/l	4.80 (4.29-5.40)	5.32 (4.56-6.36)	0.013 ^a
Triglycerides, mmol/l (median)	1.16 (0.79-1.39)	1.12 (0.82-1.55)	0.504

Data are means ± sd, number (%) or median (interquartile range). HbA1c: glycated hemoglobin; GFR: glomerular filtration rate, HDL: high density lipoprotein. Data were compared by paired samples t-test. ^aP<0.05.

Magnetic resonance imaging of the brain

Brain grey and white matter volumes were smaller in T1DM (**Table 2**). Both the thalamus ($p<0.001$) and the hippocampus ($p=0.003$) were significantly smaller in T1DM. Of the basal ganglia, volumes of the nucleus accumbens ($p=0.044$), the globus pallidus ($=0.013$), the putamen ($p<0.001$) and the nucleus caudate ($p=0.044$) were smaller in patients with T1DM compared to healthy volunteers. The amygdala was the only structure of the grey matter areas explored in this study which did not differ in size between groups ($p=0.984$).

After correcting for the potential confounding factors age, gender, GFR, smoking, BMI, hypertension, HDL-cholesterol and triglycerides, the volumes of the thalamus ($p=0.034$), hippocampus ($p=0.010$) and putamen ($p=0.002$) remained significantly smaller in T1DM (**Table 2**). HbA1c was not associated with volumes of any of the grey matter areas.

Table 2. Magnetic resonance imaging characteristics of patients with type 1 diabetes mellitus and healthy controls

	Diabetes mellitus (n=62)	Healthy controls (n=62)	P value
	Volume, cm ³	Volume, cm ³	
Nucleus accumbens	0.46 ± 0.13	0.50 ± 0.10	0.043 ^a
Globus pallidus	1.92 ± 0.25	2.02 ± 0.20	0.013 ^a
Amygdala	1.38 ± 0.23	1.38 ± 0.21	0.984
Putamen	4.77 ± 0.47	5.17 ± 0.57	<0.001 ^a
Nucleus caudate	3.57 ± 0.41	3.70 ± 0.44	0.044 ^a
Thalamus	7.53 ± 0.87	7.98 ± 0.77	<0.001 ^a
Hippocampus	3.96 ± 0.40	4.17 ± 0.41	0.003 ^a
Whole brain			
Grey matter	7.09 ± 0.45 × 10 ²	7.34 ± 0.43 × 10 ²	<0.001 ^a
White matter	7.04 ± 0.47 × 10 ²	7.24 ± 0.46 × 10 ²	0.014 ^a
Grey + white matter	14.14 ± 0.72 × 10 ²	14.57 ± 0.74 × 10 ²	<0.001 ^a

Values are means ± sd unless otherwise indicated. Volumes are corrected for head size. Grey matter, volume of normalized grey matter; White matter, volume of normalized white matter; Grey + white matter, volume of white and grey matter. Data were compared by paired samples t-test. ^a P<0.05.

DISCUSSION

The main purpose of the current study was to investigate with VBM technique whether the volumes of the hippocampus, thalamus, amygdala and basal ganglia, differed between patients with longstanding T1DM and healthy controls. The main finding of this study is that T1DM patients showed volume loss of all of these grey matter areas, except for the amygdala. Secondly our results show that after correcting for potential confounding factors, the thalamus, hippocampus and putamen were still significantly smaller in patients with T1DM.

Previous studies on hippocampal and thalamic volumes in T1DM have been performed (12,14,15,24). However, to our knowledge this is the first study to investigate in depth volume loss in grey matter areas other than the hippocampus and thalamus, in T1DM with VBM. Earlier studies on grey matter in T1DM showed equivocal results with respect to the hippocampus and thalamus (12,14,15,24). However, three of these studies were performed on MR scanners with lower field strengths 1.0T (15) and 1.5T (12,14), as compared to our 3.0T MR scanner. In addition two of these studies did not use VBM to calculate the hippocampal volumes (14,15).

In our study the amygdala was the only structure with preserved volume in T1DM patients which is in contrast to a study performed in patients with type 2 diabetes mellitus (25). The amygdala has an important role in the reaction to hypoglycemia (26). Although the mechanism behind the differential volume remains unclear, the preservation of the

volume of the amygdala could be related to its important function in the hypoglycemia awareness.

The underlying mechanisms for grey matter changes in T1DM are incompletely understood. There are several hypotheses on the pathological mechanisms causing these brain alterations. Both hyper- and hypoglycemia have been proposed to be involved. Hypoglycemic events are associated with less grey matter density (4,12,27). Furthermore hyperglycemia, expressed as a higher HbA1C, was suggested to contribute to lower grey matter density in the left hippocampus (12), which was not confirmed in our study. In addition, accelerated formation and accumulation of advanced glycation end products have also been suggested to contribute to brain alterations and brain damage in T1DM (13,28,29). It has also been proposed that diabetes mellitus contributes to accelerated aging of the brain, which can lead to microvascular abnormalities (5,13). Furthermore, prior studies suggest that higher blood pressure or hypertension also negatively affect grey matter (30-33). However, in our study, effects of T1DM on specific grey matter structures remained significant after correction for hypertension. The intriguing finding that T1DM differentially affects specific grey matter areas is challenging but difficult to explain. Potential differences in the susceptibility of the thalamus, hippocampus and putamen and other grey matter structures to T1DM related factors may be involved.

In conclusion using VBM technique, we found differential volume loss of the hippocampus, thalamus and basal ganglia in patients with T1DM, which was independent of potential confounding factors. The clinical significance and underlying mechanisms remain to be elucidated.

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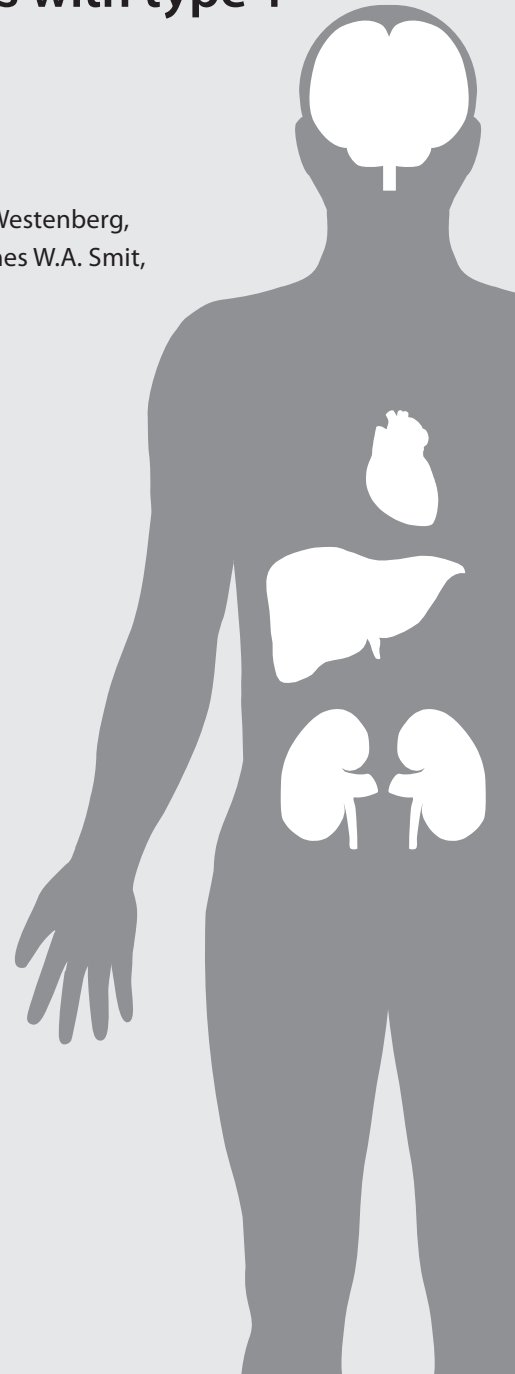
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Chapter 6

Aortic stiffness is associated with white matter integrity in patients with type 1 diabetes

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ABSTRACT

Objective

To assess the association between aortic pulse wave velocity (PWV), as a marker of arterial stiffness and diffusion tensor imaging (DTI) of brain white matter integrity in patients with type 1 diabetes using advanced magnetic resonance imaging (MRI) technology.

Methods

Forty-one patients with type 1 diabetes (23 men, mean age 44 ± 12 years, mean diabetes duration 24 ± 13 years) were included. Aortic PWV was assessed using through-plane velocity-encoded MRI. Brain DTI measurements were performed on 3 Tesla MRI. Fractional anisotropy (FA) and apparent diffusion coefficient (ADC) were calculated for white and grey matter integrity. Pearson correlation and multivariable linear regression analyses including cardiovascular risk factors as covariates were assessed.

Results

Multivariable linear regression analyses revealed that aortic PWV is independently associated with white matter integrity FA (Beta= -0.777, $p=0.008$) in patients with type 1 diabetes. This effect was independent of age, gender, mean arterial pressure, body mass index, smoking, duration of diabetes and glycated hemoglobin levels. Aortic PWV was not significantly related to grey matter integrity.

Conclusions

Our data suggest that aortic stiffness is independently associated with reduced white matter integrity in patients with type 1 diabetes.

INTRODUCTION

Type 1 diabetes is a common chronic metabolic disorder, which is associated with diverse comorbidity. Although diabetes mellitus is not primarily a brain disease it may be associated with cerebral atrophy, white matter hyperintensities and infarctions as well as decreased cognitive functioning [1-3].

Besides well-recognized risk factors in patients with type 1 diabetes, like chronic hyperglycemia and alterations in insulin metabolism, vascular risk factors are thought to contribute to the occurrence of brain abnormalities. Aortic stiffening may play a central role in the development of brain injury. Of note, previous studies have shown that aortic stiffness is associated with generalized white matter atrophy and cerebral small vessel disease in type 1 diabetes [4, 5]. Aortic stiffness may contribute to microvascular brain injury by exposing the small vessels to the high pressure fluctuations and flow in the cerebral circulation [6]. The brain is vulnerable in particular to high pressure fluctuations, because it is perfused at high volume flow and has very low vascular resistance. Pulse wave velocity (PWV) is a widely used surrogate marker for arterial stiffness. It is defined as the propagation speed of the systolic flow wave front traveling along the aorta, reflecting the elastic properties of the aortic vessel wall. PWV is estimated by dividing the distance between anatomical locations over the aorta by the time difference between the arrival of flow waves at the two locations. Higher PWV (m/s) corresponds to higher arterial stiffness [7].

Diffusion tensor imaging (DTI) is a validated technique of brain imaging, which, in contrast to conventional methods, enables measurement of white matter microstructure [8]. DTI is based on diffusivity of water, providing two parameters of tissue integrity, namely the apparent diffusion coefficient (ADC) which reflects the magnitude of diffusion and fractional anisotropy (FA) which is a measure of the directionality of the diffusion and flow of water molecules in the brain tissue. Early detection of tissue injury reflected by an increase ADC and reduction in FA has been observed in normal aging and cerebral small vessel disease [1]. Moreover, Kodl et al. showed white matter microstructural deficits in patients with longstanding type 1 diabetes using this technique [8].

Interestingly, increased pulse pressure, an indicator of arterial stiffness, was found to be associated with lower FA in healthy volunteers [9]. There are no previous publications that explored the possible relationship between aortic stiffness and DTI of the brain in patients with type 1 diabetes using well-validated MRI techniques. Accordingly the purpose of this study was to assess the association between aortic pulse wave velocity, and DTI of brain white matter integrity in patients with type 1 diabetes using advanced MRI technology.

MATERIALS AND METHODS

Study participants

This study was approved by the local medical ethics committee and all subjects gave written informed consent. Between February 2008 and January 2010 consecutive patients with type 1 diabetes were recruited from the local outpatient clinic. Characteristics of the study population are detailed in **Table 1**. Forty-one patients with type 1 diabetes (23 men and 18 women) were included with a mean age of 44 ± 12 years. Mean duration of the type 1 diabetes was 24 ± 13 years. All patients were treated with insulin. One patient was using an ACE-inhibitor in combination with an angiotensin II-antagonist for the presence of albuminuria. None of the other patients with type 1 diabetes were using blood pressure lowering medication. Statins were used by 11 (26%) patients.

Information about the characteristics of type 1 diabetes was obtained by standardized interviews and physical and laboratory examinations. Exclusion criteria for all participants were congenital aortic/heart disease, a clinical history of hypertension or cardiovascular disease, evidence of aortic valve stenosis or insufficiency, any other systemic disease than diabetes and general contraindications to MRI. At MRI examination duration of diabetes in years was estimated from self-reported year of diagnosis. Blood pressure and heart rate were measured using a semiautomated sphygmomanometer (Dinamap, Critikon, Tampa, Fla, USA) after MR imaging. Pulse pressure was defined as systolic blood pressure minus diastolic blood pressure. Mean arterial pressure (MAP) was estimated by adding up one-third of the pulse pressure to diastolic blood pressure. Body mass index and smoking status (nonsmoker or current smoker) were determined. High-density lipoprotein (HDL), total cholesterol, triglycerides, creatinine and glycated hemoglobin (HbA1C) were also determined.

This patient group was partly included in a previous study describing the predictive value of cerebral blood flow and aortic stiffness on generalized white matter brain atrophy in type 1 diabetes [4].

MRI protocol

MR imaging of the aorta and brain were performed at the same day. Aortic imaging was performed, as previously described, using a 1.5T MRI scanner (NT 15 Gyroscan Intera; Philips Medical Systems, Best, the Netherlands) [10]. In short: a scout image of the aorta was acquired using a five-element phased array cardiac surface coil. Aortic PWV was assessed according to the transit-time method from two one-directional through-plane velocity-encoded MRI acquisitions with high-temporal resolution performed in the aorta at two predefined levels: at the level of the pulmonary trunk in the ascending and in the abdominal descending aorta [10]. Scan parameters were: repetition time (TR) 5.0ms, echo time (TE) 2.9ms, flip angle (FA) 20°, field of view (FOV) 300mm, 128×115 acquisition

matrix, slice thickness 8mm, with maximal number of phases reconstructed ensuring high (6-10ms) effective temporal resolution. Aortic velocity maps were analyzed using the in-house developed FLOW software package.

Table 1. Clinical characteristics and MR findings of patients with type 1 diabetes

	Patients with type 1 diabetes (n=41)
Clinical characteristics	
Age, years	44 ± 12
Male gender, n (%)	23 (55)
Body mass index, kg/m ²	25.3 ± 2.9
Systolic blood pressure, mmHg	128 ± 19
Diastolic blood pressure, mmHg	73 ± 10
Pulse pressure, mmHg	55 ± 14
Mean arterial pressure, mmHg	91 ± 12
Heart rate, beats/min	66 ± 10
Current smoker, n (%)	6 (15%)
Alcohol use yes, n (%)	23 (56%)
Laboratory markers	
HbA1c, %	7.6 ± 1.0
HDL-cholesterol, mmol/l	1.7 ± 0.5
Total cholesterol, mmol/l	4.8 ± 1.0
Triglycerides, mmol/l	1.2 ± 0.6
Creatinine, umol/l	75 ± 11
MRI findings	
Aortic PWV, m/s (median, IQR)	6.2 (5.2-7.6)
Stroke volume, ml/heart beat (median, IQR)	87 (81-105)
Periventricular WMHs, n (%)	7 (17)
Subcortical WMHs, n (%)	2 (5)
Grey matter brain volume, mL	574 ± 60
Grey matter FA	0.171 ± 0.008
Grey matter ADC	0.00042 ± 0.00002
White matter brain volume, mL	564 ± 69
White matter FA	0.344 ± 0.017
White matter ADC	0.00036 ± 0.00001
Total brain volume, mL	1139 ± 121
Total brain FA	0.252 ± 0.010
Total brain ADC	0.00040 ± 0.00001

Data are presented as mean and standard deviations unless otherwise stated. BMI: body mass index; HbA1c: Glycated hemoglobin, HDL: high-density lipoprotein, PWV: pulse wave velocity, IQR: inter quartile range, FA: fractional anisotropy, ADC: apparent diffusion coefficient.

The aortic PWV is defined by the propagation of the systolic flow wave front, and was determined by the distance between the aortic measurement sites and the transit-time of the systolic flow wave front (**Figure 1**). This transit-time is assessed by the time difference in arrival of the systolic flow wave front at each level, automatically assessed by definition of the intersection between the horizontal diastolic flow and the upslope of the flow wave, modeled by linear regression of all data points between 20% and 80% of the range of flow values along the slope. The aortic path length between the measurements sites was measured by manually drawing a centerline in the aorta as defined in the aortic scout view, using the in-house developed software package MASS (Medis, Leiden, the Netherlands). Aortic vessel wall contour segmentation was performed by a researcher unaware of the subjects' conditions and obtained data with brain MRI. **Figure 2** shows the flow chart for the processing data.

For the assessment of systolic LV function, the LV was imaged in short-axis orientation as previously described [11] by using a retrospectively-gated breath-hold segmented gradient-echo sequence with steady-state free-precession. Ten to twelve consecutive slices of 10mm thickness and without gap were obtained with one signal average and 40 reconstructed phases. Scan parameters: echo time TE 1.7 ms, repetition time TR 3.3 ms, flip angle 50°, acquisition matrix 256×194, acquisition voxel size 1.7×1.7×10mm³.

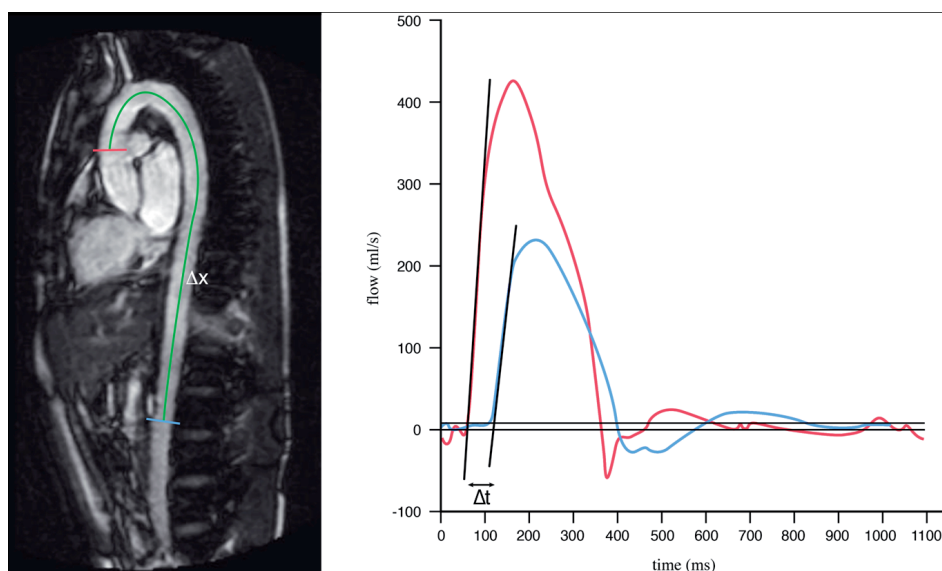


Figure 1. Aortic pulse wave velocity assessment. The left panel shows an oblique sagittal scout view of the aorta. Velocity mapping was obtained at the level of the pulmonary trunk and in the abdominal descending aorta. The aortic path length (Δx) between both levels was measured, indicated by the polyline following the centerline of the aorta. The right panel illustrates the flow curves in the ascending (red line) and descending (blue line) aorta. The transit time of arrival of the pulse wave (Δ) was divided by the aortic path length (Δ) to calculate aortic PWV (in meters per second).

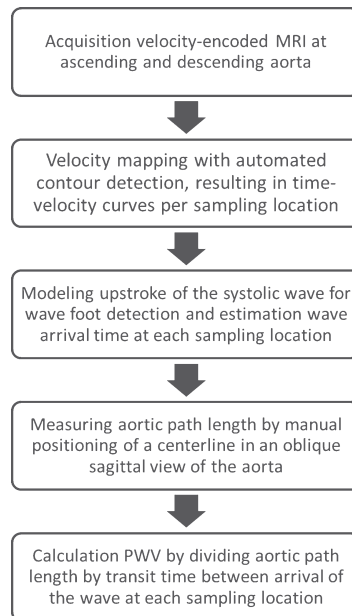


Figure 2. Flow chart of the processing of pulse wave velocity

Using the software package MASS, end systolic and end diastolic LV volumes were obtained using manual contour definition of the endocardial borders in these multi-slice and multi-phase datasets. Left ventricular end-systolic and end-diastolic volume, cardiac output and stroke volume were assessed [12].

All brain MRIs were performed on a 3.0 Tesla (Achieva; Philips Medical Systems, Best, The Netherlands). Brain MRI consisted of a 3 dimensional T1 (3D T1) sequence for brain volume assessment. DTI was performed to assess fractional anisotropy (FA) and apparent diffusion coefficient (ADC). 3D T1 acquisition parameters were: TR 9.8ms, TE 4.6 ms, FA 8°, FOV 224mm, 192×152 acquisition matrix, 256×256 reconstruction matrix, slice thickness 1.2mm, 120 slices, no slice gap). For DTI, a single-shot echo-planar sequence was applied with 32 measurement directions having the following scan parameters: TR 10,004ms, TE 56ms, FOV 220×220×128 with an acquisition matrix of 112×110, 2.00mm slice thickness, transversal slice orientation, slice gap = 0, FA 90°, single reconstruction voxel dimensions were 1.96×1.96×2.00mm, number of slices = 64, B factor = 1,000, halfscan factor = 0.61. Parallel imaging (SENSE) was used with a reduction factor of 2, NSA = 1 and fat suppression was applied. DTI acquisition time was 6.55min.

MRI post processing

Evaluation of cerebral small vessel disease was evaluated on a spin-echo T2-weighted and fluid-attenuated inversion recovery (FLAIR) sequences. White matter hyperintensi-

ties (WMHs) were defined as areas of brain parenchyma with increased signal on T2-weighted and FLAIR images without mass effect. Lacunes defined as areas of more or less complete focal tissue destruction are not included in the rating of WMHs. WMHs were classified according to Fazekas et al. [13, 14] and subdivided into either periventricular or subcortical. Periventricular lesions were defined as starting directly at the ventricular border. When these abnormalities extend > 1cm into the adjacent white matter, this by convention indicates damage to both the periventricular and deep white matter. Deep WMH are characterized by a rim of normal appearing tissue which separates them from the periventricular region. Subjects were divided into those with normal [0] vs abnormal [1] amounts of WMHs. Fazekas scores of 0 and 1 were considered normal, a score of 2 was considered abnormal below the age of 75 years, and a score of 3 was considered abnormal in any age group.

All T1-weighted scans were analyzed using software provided by FMRIB's Software Library (FSL) [15]. Total brain tissue volume was estimated with SIENAX [16, 17]. This program extracts brain and skull images from the single whole-head input data [18]. The brain images were then affine-registered to MNI152 space, using the skull images to determine the registration scaling with 7 degrees of freedom (FSL-FLIRT. FSL's FAST was used to create GM and WM tissue segmentations. A visual inspection of all registrations was performed and of the final segmentations 20 % were selected at random and visually inspected for accuracy. ADC and FA reconstructions were performed direct after data acquisition using software provide by the manufacturer (Philips Medical Systems, Best, The Netherlands). To correct for possible partial volume effects in ADC and FA data, especially from CSF, an eroded mask of GM and WM segmentations was created by removing one voxel in plane for all above named VOIs. FA and ADC reconstructions were then registered to the eroded whole brain, WM and GM volumes, using the same transformation matrix obtained from the original linear registration of the T1-w volume to MNI space. Finally, mean ADC and FA values were calculated for (eroded) whole brain, (eroded) WM and (eroded) GM. The researcher who obtained the FA and ADC data was unaware of the subjects' conditions and PWV data .

Statistical analysis

Statistical analysis was performed with SPSS (SPSS, Chicago, Illinois, USA), version 17. Continuous variables are expressed as mean $\pm$ standard deviation (sd) unless otherwise stated. Categorical variables were presented as frequencies and percentages. Aortic PWV and stroke data were non-normally distributed and therefore were log-transformed for further analyses. Regression analysis, corrected for age and gender was used to test whether subjects with white matter hyperintensities have a different PWV than subjects without. The correlation between aortic PWV and between stroke volume with brain integrity was tested by Pearson correlation analysis. Multivariate linear regression analy-

ses were performed to adjust for confounding factors, defined as age, gender, mean arterial pressure, body mass index, smoking, duration of diabetes and HbA1c. These covariates were entered simultaneously into a multivariable linear regression model. The β -regression coefficients and p-values are reported. Significance was reached when $p < 0.05$ (two-tailed).

RESULTS

Aortic PWV and brain integrity

PWV did not differ between patients with or without periventricular WMHs ($p=0.250$) but was higher in subjects with subcortical WMHs (Beta 0.235, $p=0.008$). There were significant correlations between the aortic PWV and white matter integrity FA ($r=-0.529$, $p < 0.001$) and ADC ($r=0.406$, $p=0.009$). The correlation between PWV and white matter FA is shown in **Figure 3**. For grey matter integrity a significant correlation was found for ADC ($r=0.518$, $p=0.001$) but not for FA ($r=0.016$, $p=0.923$). Stroke volume correlated with grey matter integrity FA ($r=0.342$, $p=0.029$) and ADC ($r=-0.368$, $p=0.018$) and with white matter FA ($r=0.326$, $p=0.038$). Multivariable linear regression analyses showed that aortic PWV is independently associated with WM integrity expressed as FA (Beta=-0.777, $p=0.008$) in patients with type 1 diabetes (**Table 2**). This effect was independent of age, gender, MAP, BMI, smoking, duration of diabetes and HbA1c levels. In a similar multivariable linear regression model for the prediction of grey matter (GM) integrity no significant effect of aortic PWV was observed. Lipid lowering drugs (used by 26% of the patients) may reduce arterial stiffness. Therefore further adjustment for the use of statins was performed but this did not yield different results for the association between PWV

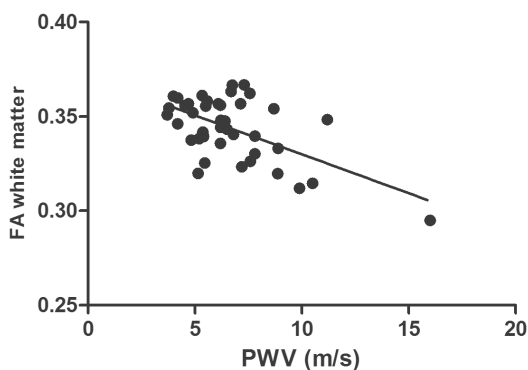


Figure 3. Correlations between the log-transformed pulse wave velocity (lnPWV) and fractional anisotropy (FA), a DTI parameters of the white matter.

Table 2. Results of multivariable linear regression analyses performed in patients with type 1 diabetes (n=41) to assess independent predictors of WM and GM integrity

	White matter integrity				Grey matter integrity			
	ADC		FA		ADC		FA	
	Beta	p-value	Beta	p-value	Beta	p-value	Beta	p-value
Age, years	-0.068	0.859	0.111	0.731	0.252	0.444	-0.157	0.700
Female gender (n=19)	-0.166	0.418	0.123	0.474	-0.230	0.192	0.091	0.676
Mean arterial pressure, mmHg	-0.048	0.830	0.064	0.731	-0.245	0.202	0.044	0.850
Body mass index, kg/m2	0.079	0.666	-0.057	0.712	0.039	0.804	-0.100	0.607
Current smoker	-0.037	0.831	0.149	0.319	-0.015	0.922	0.253	0.182
Duration of diabetes, years	-0.078	0.753	-0.058	0.781	0.313	0.146	-0.052	0.844
HbA1c, %	-0.112	0.600	-0.011	0.949	-0.166	0.363	-0.203	0.357
Aortic PWV, m/s	0.532	0.111	-0.777	0.008*	0.270	0.338	-0.098	0.778

ADC: apparent diffusion coefficient, FA: fractional anisotropy, HbA1c: Glycated hemoglobin, PWV: pulse wave velocity. * p-value < 0.05.

and WM integrity (FA Beta=-0.798, p=0.006, ADC Beta=0.555, p=0.203). No significant association between stroke volume and integrity was found in multivariable linear regression analyses.

DISCUSSION

The main finding of our study is the observation, that aortic stiffness is associated with white matter integrity independently of other potential confounders in patients with type 1 diabetes.

So far few studies have reported on white matter integrity in type 1 diabetes. White matter microstructural deficits have been observed in patients with type 2 diabetes [19, 20] independent of age and blood pressure levels. Kodl et al. showed white matter microstructural deficits expressed as reduced FA in 25 patients with longstanding type 1 diabetes, as compared to 25 age and gender matched healthy volunteers [8]. The patients in our study have a similar age and a somewhat shorter duration of diabetes (24±13 years vs. 30±11 years).

As hypothesized we found that arterial stiffness was associated with white matter microstructure expressed as reduced FA in patients with type 1 diabetes. The association with PWV was not found for ADC. Possibly this may be due to the fact that ADC reflects general diffusional properties, therefore being a more heterogeneous white matter measure than FA, which reflects organizational aspects. Crucial in this analysis was to incorporate age as a covariate as it is known that measures of diffusion such as FA and ADC, as well as arterial stiffness are dependent on age [19, 21]. Aortic PWV

is a surrogate marker for arterial stiffness and has been shown to be an independent predictor for stroke, cerebral small vessel disease [5, 6, 20, 21] and might be a predictor for cognitive decline [22]. Furthermore in type 1 diabetes aortic PWV measured with MRI has been demonstrated a predictor of white but not grey matter atrophy [4]. In line with this study we could not show an independent association for aortic PWV with grey matter integrity.

It is known that the blood flow in the white matter is substantially lower compared to the blood flow in grey matter. White matter may be more susceptible to subtle pressure fluctuations than grey matter, because of the vulnerable end-arterioles penetrating the white matter [23]. It has been hypothesized that in particular “watershed” areas of the brain may be sensitive to arterial stiffness [24]. In line, PWV has been associated with processing speed and memory, cognitive functions represented in these watershed locations [25]. To the best of our knowledge we are the first to show an association between PWV and white matter integrity in patients with type 1 diabetes as evaluated by MRI techniques. In agreement with our study, a relation between pulse pressure, another marker of arterial stiffness, and white matter deficits has been observed [9]. However that relationship was studied in healthy normotensive subjects. Furthermore aortic PWV is considered to be a more accurate method for assessing arterial stiffness than the measurement of pulse pressure made in the peripheral vessels which does not always accurately reflect the actual central pulse pressure and which may be unreliable in older subjects.

In diabetes mellitus both glucose toxicity and abnormal insulin metabolism have been suggested to contribute to brain pathology [26, 27]. Up to now few studies investigated the relationship between clinical parameters of disease severity and microstructural white matter changes in diabetes. In patients with type 2 diabetes disease duration rather than HbA1C was related to DTI assessed white matter changes when investigated simultaneously [19]. Interestingly we did not observe associations between white matter microstructure and clinical parameters such as disease duration or HbA1c. This is in contrast to an earlier study that identified a significant relationship between FA and HbA1c and between FA and disease duration in type 1 diabetes. However in that study no adjustments for age were performed. Furthermore those authors did not report on blood pressure while we investigated patients with type 1 diabetes without a history of hypertension. Since high blood pressure was shown to be associated with a decrease in FA [28] and is frequently occurring in diabetes it is not clear to which degree this may have influenced these previous results.

MR-DTI is a technique that allows early detection of white matter damage even in regions that appear normal on conventional anatomical images. Despite a limited understanding the exact pathophysiology, DTI changes could be of clinical relevance. In children DTI assessed structural integrity is associated with higher IQ and loss of

integrity in normal aging is associated with cognitive decline [29, 30]. In addition in patients with type 1 diabetes white matter deficits were associated with performance on neurocognitive tests [8]. In order to develop targeted treatments to prevent brain damage it is important to understand the underlying mechanisms that cause subtle brain alterations. Our findings support the role of vascular mechanisms leading to a decline in white matter microstructure in type 1 diabetes, which adds to current knowledge.

Smoking is known to increase arterial stiffness [31-33] consequently we adjusted for smoking in the linear regression analyses. Alcohol consumption may have a divergent effect on arterial stiffness and may also directly affect the brain, however as the number of alcohol units were not registered we were not able to explore the effect of alcohol. Furthermore this was an explorative study and associations already detected in this small patient group need to be confirmed in a larger population. Given the cross-sectional design of this study no insight into the changes over time or a causal relationship between aortic PWV and white matter damage can be determined. Another limitation of our study is that we did not include cognitive testing in the current study. Longitudinal studies are needed to assess the impact of our findings on cognitive decline and to assess the prognostic and therapeutic implications.

In conclusion we found that aortic stiffness is independently associated with reduced white matter integrity in patients with type 1 diabetes, which suggests a vascular contribution to early subtle microstructural deficits. Future prospective studies are needed to improve knowledge of the prognostic and therapeutic implications of these microstructural changes.

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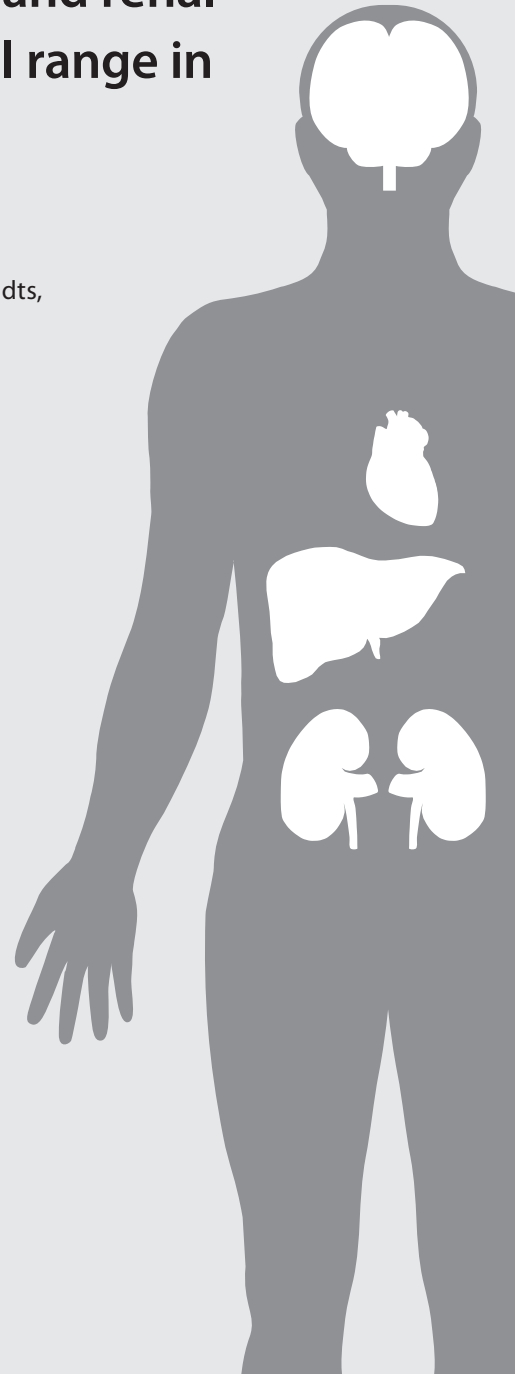
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Chapter 7

Relation between MR assessed regional aortic pulse wave velocity and renal function within the normal range in hypertensive patients

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Submitted



ABSTRACT

Purpose

To assess the association between magnetic resonance (MR) imaging assessed regional aortic PWV and renal function within the normal range in patients with hypertension.

Materials and Methods

In this cross-sectional study, 96 patients with hypertension and preserved renal function and 44 healthy volunteers (mean age 46 ± 13 years and 44 ± 15 years) were included. Renal function was expressed as estimated glomerular filtration rate (eGFR). PWV was assessed in the total, proximal and distal part of the aorta by velocity-encoded MR imaging.

Results

An increased PWV for both aortic segments as well as for the total aorta was found in patients with hypertension compared to healthy volunteers [PWV_{total} 6.5 vs 5.5 m/s, $PWV_{proximal}$ 6.1 vs 5.6 m/s, PWV_{distal} 6.6 vs 5.4 m/s, $p \leq 0.028$ and 0.03]. The ratio between $PWV_{distal} : PWV_{proximal}$ was not significantly different between groups. There was a statistically significant correlation between eGFR and aortic PWV in both patients with hypertension ($r = -0.380$, $p < 0.001$) and healthy volunteers ($r = -0.383$, $p = 0.010$). The decrease in eGFR per increase in PWV of the total aorta was not significantly different between both groups ($p = 0.783$), however patients with hypertension showed a higher aortic PWV for each given eGFR ($p = 0.047$).

Conclusion

Changes in renal function within the normal range are inversely related to increased proximal and distal aortic stiffness even in the absence of disproportional stiffening. This relationship is present before the onset of proteinuria and macrovascular complications. These results stress the close link between aortic stiffness and the microcirculation.

INTRODUCTION

Arterial stiffness measured as pulse wave velocity (PWV) is an independent predictor of cardiovascular disease in hypertension as well as chronic renal disease (1). Hypertension is an important determinant of aortic wall stiffening due to the continuous and repetitive pulsatile stress acting on the arterial wall which causes fracturing of the elastin and collagen network. A close link between arterial stiffness and microvascular renal disease exists and this relation appears to be bidirectional. Renal dysfunction may contribute to stiffening by numerous mechanisms including vascular calcification, activation of the renin-angiotensin-aldosterone system, chronic inflammation and endothelial dysfunction (2).

Contrawise arterial stiffening of the aortic wall may promote renal microvascular damage. Accordant with local variation in structure, arterial stiffening from aging or hypertension varies along the arterial tree, with increased stiffening of the central elastic arteries compared to more muscular distal arteries. Disproportionate arterial stiffening may accelerate transmission of excessive pulsatile energy toward the microcirculation (3). As a result the hypertensive microcirculation may not be exposed to the same pulsatile energy than in normotension. In particular end-organs with high-flow and low vascular resistance, such as the brain and kidneys, are predisposed to the damaging effects of pressure pulsatility.

Recently disproportionate stiffening of the proximal aorta compared to the carotid arteries was shown to be associated with microvascular structural brain damage (4,5). Interestingly this was associated with a reduction in blood flow volume towards the brain. It was hypothesized that vascular resistance in response to elevated pulse pressure will reduce mean blood flow. The resulting mismatch may contribute to microvascular damage in the brain. Likewise, age or hypertension related decline in renal function may be explained. Evidence supporting the hypothesis that aortic stiffness may contribute to lower glomerular filtration rate by transferring excessive flow pulsatility into the renal microcirculation was provided by the AGES study. In this study the relation between aortic PWV was mediated by higher renal artery pulsatility index and lower arterial volume to the renal cortex (6).

However like most clinical studies which relate arterial stiffness to abnormalities in kidney function, generalization of the results was limited by the studied population. Nearly all studies have been performed in elderly subjects, subjects with end-stage renal disease or mild renal dysfunction. In patients with hypertension arterial stiffening as well as impaired renal function often co-exist. Therefore, whether increased PWV in patients with hypertension is directly related to high blood pressure or depends primarily on co-existing renal dysfunction remains to be elucidated. Moreover there are no previous publications that explored the possible relationship between regional aortic

stiffening and renal function within the normal range in patients with hypertension. Magnetic resonance imaging (MRI) in combination with velocity-encoding (VE) is a well-validated technique that enables assessment of PWV in specific segments of the aorta. Our hypothesis is that increased aortic stiffness contributes to lower glomerular filtration rate in the normal range by transferring elevated pulse pressure and is possibly mediated by disproportional aortic stiffening. Accordingly, the purpose of this study was to assess the relation between regional aortic PWV and renal function in the normal range in patients with hypertension using VE-MRI.

METHODS

Study participants

For this study a total 96 consecutive patients with hypertension were recruited from the outpatient clinic of the Leiden University Medical Center. Healthy volunteers (n=44) without a history or clinical evidence of diabetes mellitus, hypertension or cardiovascular disease were recruited by advertisement in local newspapers. Exclusion criteria consisted of aortic valve stenosis or insufficiency, Marfan syndrome, aortic coarctation or any aortic disease, established cardiovascular disease, pregnancy and standard MRI contra-indications. All subjects signed informed consent after a detailed description of the procedure. For this study 15 patients were excluded because of impaired renal function defined as an estimated glomerular filtration rate (eGFR) below 60 ml/min/1.73² and no proteinuria (<300 mg/24h urine). Part of this patient group was included in a previous study describing the relation between aortic stiffness, left ventricular mass and lacunar brain infarcts in hypertension (7).

Blood pressure was measured after MRI using a semi-automated sphygmomanometer (Dinamap, Critikon, Tampa, FL, USA). Hypertension was diagnosed after repeated blood pressure measurements in sitting position according to the guidelines of the European Society of Hypertension (8). Duration of hypertension was estimated as time (in years) from year of magnetic resonance (MR) imaging examination minus self-reported year of diagnosis. Pulse pressure was defined as systolic blood pressure minus diastolic blood pressure. Body mass index (BMI) was determined, smoking status was obtained and participants were divided into current smokers and non-smokers. Information about diabetes mellitus and the current use of medication was obtained from medical history and medical status of the outpatient clinic. After an overnight fast and within two weeks before MR imaging a blood sample was drawn for determination of creatinine, glucose and lipid profiles (total cholesterol, high density lipoprotein and triglycerides).

The Modification of Diet in Renal Diseases (MDRD) equation was used to calculate renal function which provides an accurate GFR estimate using serum creatinine (9). The

study was conducted according to the principles in the Declaration of Helsinki and the study was approved by the local medical ethics committee.

MR imaging

A 1.5 Tesla MRI (NT 15 Gyroscan Intera; Philips Medical Systems, Best, the Netherlands) was used for the evaluation of aortic stiffness using a previously described protocol (10). In short, on multi-oriented scout images of the aorta, two imaging planes were planned perpendicular to the aorta: one to the ascending aorta at the level of the pulmonary trunk, transecting both the ascending and proximal descending aorta, and one at the level of the abdominal descending aorta 7.5 cm beneath the diaphragm (**Figure 1**). At these planes, one-directional through-plane velocity-encoded (VE) MRI with retrospective ECG-gating was used. Maximal velocity sensitivity for the first plane was set at 150 cm/s, and for the second plane 100 cm/s. At each cross-section of the aorta, blood flow velocity-time curves were assessed by velocity mapping. Aortic PWV was calculated as the aortic path length between consecutive sampling sites (i.e., at ascending, proximal descending and abdominal aorta) divided by the time duration for the systolic flow velocity wave to propagate between sampling sites (**Figure 1**). The time point of wave

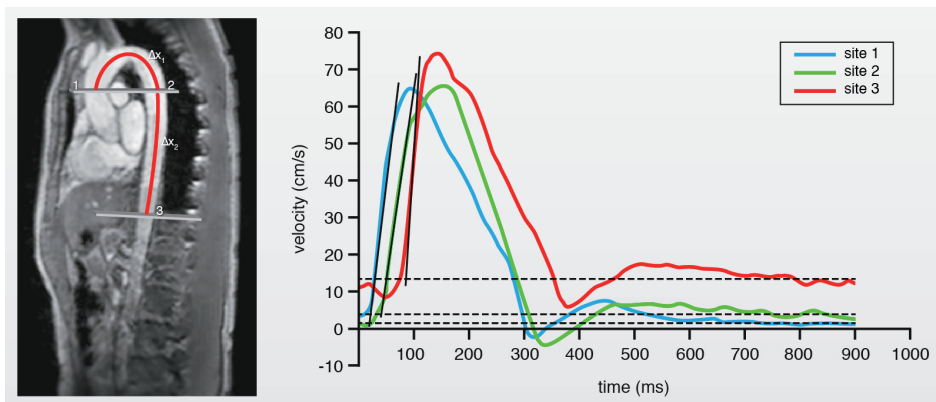


Figure 1. Assessment of aortic pulse wave velocity

On the left, an oblique sagittal scout view of the proximal and distal aorta is shown. Two imaging planes were planned perpendicular to the aorta: one to the ascending aorta at the level of the pulmonary trunk, transecting both the ascending and proximal descending aorta, and one at the level of the abdominal descending aorta 7.5cm beneath the diaphragm. The aortic path length (Δx_1 for the ascending aorta, Δx_2 for the abdominal aorta) between these levels was measured indicating by the red line following the centerline of the aorta. On the right, the flow curves in ascending and abdominal aorta are illustrated. The horizontal dashed lines represent the horizontal diastolic flow at the corresponding site in the aorta. The line following the upstroke is determined by linear regression of all data point between 20% and 80% of the range of values along the slope. Time point of intersection was considered being the arrival time of the pulse wave. Aortic PWV was calculated as the aortic path length between consecutive sampling sites divided by the time duration for the systolic flow velocity wave to propagate between sampling sites.

arrival was assessed from automated detection of the foot of the pulse wave, from the transection of two lines: one modeling the horizontal diastolic flow from the last 250 ms of the velocity-time curve and one modeling the upslope of the wave by linear regression of all data point between 20% and 80% of the range of values along the slope (11). From these data PWV for the proximal aorta (ascending and proximal descending aorta), PWV for the distal aorta (descending and abdominal aorta) and total aorta was calculated. Data were analyzed with MASS/Flow (Leiden University Medical Center, Leiden, the Netherlands). Analyses were performed by one researcher (A.B. with 1 year of experience in cardiac MR imaging) and supervised by a senior researcher (J.J.M.W. with 13 years of experience in cardiac MR imaging).

Statistical analysis

An independent samples t-test for continuous variables, Mann-Whitney U test for non-normally distributed variables and Chi-square test for dichotomous variables were used to compare clinical characteristics between patients with hypertension and healthy volunteers. Since aortic PWV was non-normally distributed (Kolmogorov-Smirnov $p < 0.001$), the correlation between aortic PWV and renal function was tested by Spearman correlation and a log transformation of aortic PWV values was used for regression. Multivariable regression with forward entry analysis for eGFR was carried out to study the relation between aortic PWV and the effect of hypertension. To test whether the relationship between eGFR and aortic PWV differed between patients with hypertension and healthy volunteers an interaction term for the presence of hypertension was used. Furthermore, the possible confounding effect of age, diabetes mellitus, blood pressure, BMI, smoking, hypertension duration, medication using eGFR values uncorrected for age was tested. P value < 0.05 was considered statistically significant. SPSS for Windows (version 16.0; SPSS, Chicago, Illinois, USA) was used for statistical analysis.

RESULTS

Clinical, laboratory and MRI derived parameters of hypertensive patients (mean age: 46 ± 13 years) and healthy volunteers (mean age: 44 ± 15 years) are summarized in **Table 1**. Blood pressure and pulse pressure were significantly higher in patients with hypertension compared to healthy volunteers despite the use of antihypertensive drugs. Calcium channel blockers were used in 35% of the patients, renin-angiotensin system inhibitors (angiotensin receptor blockers, angiotensin converting enzyme inhibitors or rennin inhibitors) in 58% of the patients, adrenergic receptor blockers (alpha-1 or beta-blockers) in 39% of the patients, diuretics and other compounds in 31% respectively 2% of the hypertensive patients. Ten (10%) hypertensive patients were also diagnosed with

Table 1. Clinical characteristics and MRI results of the study population

	Patients with hypertension (n=96)	Healthy volunteers (n=44)	P-value
Male gender, n (%)	41(42)	23(52)	0.292
Age (years)	46±13	44±15	0.664
Hypertension duration (years)	7±(8)	-	-
Current smoker, n (%)	17(18)	4(10)	0.178
Body mass index (kg/m ²)	25.8±4.0	26.7±8.3	0.481
Systolic blood pressure (mmHg)	152±25	131±15	<0.001*
Diastolic blood pressure (mmHg)	89 ±14	81±10	<0.001*
Pulse pressure (mmHg)	63±20	50±11	<0.001*
Total cholesterol (mmol/l)	5.90±1.14	5.35±1.19	0.220
Triglycerides (mmol/l), median IQR	1.38 (1.02-1.95)	1.19 (0.84-1.69)	0.059
HDL cholesterol (mmol/l), median IQR	1.39 (1.16-1.78)	1.59 (1.36-1.81)	0.039*
Fasting glucose (mmol/l)	5.5±1.4	4.9±0.6	0.008
Estimated GFR (ml/min/1.73 ²)	85±16	83±12	0.527
<i>Aortic stiffness</i>			
Aortic pulse wave velocity (m/s), median IQR			
PWV _{proximal}	6.1 (5.3-7.7)	5.6 (4.5-7.2)	0.028
PWV _{distal}	6.6 (5.4-8.1)	5.4 (4.4-6.7)	<0.001
PWV _{total}	6.5 (5.4-7.5)	5.5 (4.5-6.8)	0.001
PWV-ratio (PWV _{distal} :PWV _{proximal})	1.0 (0.8-1.3)	0.9 (0.8-1.1)	0.064

Data are presented as mean and standard deviation unless otherwise stated. HDL = high-density lipoprotein, GFR = glomerular filtration rate *P<0.05

type 2 diabetes mellitus. Two of them were treated with insulin, others were treated with lifestyle advice (n=3) or oral glucose lowering medication (n=5). Furthermore HDL-cholesterol levels were significantly lower [1.39 (1.16-1.78) vs. 1.59 (1.36-1.81) mmol/l, p=0.039] in hypertensive patients compared to healthy volunteers. Renal function did not differ between groups.

Aortic pulse wave velocity and renal function

PWV was significantly higher in patients with hypertension compared to healthy volunteers for both segments of the aorta as well as for the total aortic PWV. PWV-ratio (PWV_{distal}:PWV_{proximal}) was not significantly different between groups.

Statistically significant correlations between total aortic PWV and eGFR both in patients with hypertension (r= -0.380, p<0.001, corresponding regression line: eGFR=-21xln(aortic PWV) + 125) and in healthy controls (r= -0.383, p=0.010, corresponding regression line: eGFR=-18xln(aortic PWV) + 115) were observed, which are shown in **Figure 2**. Accordingly significant correlations between PWV of the proximal and distal aorta and eGFR were found (**Table 2**). Patients with hypertension showed a higher

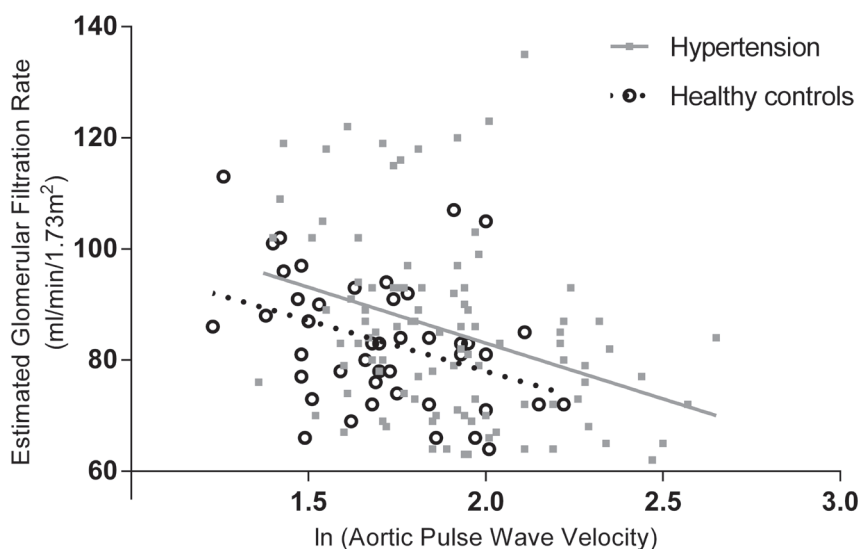


Figure 2. Correlations between aortic PWV and eGFR both in patients with hypertension ($r = -0.380$, $p < 0.001$, corresponding regression line; $eGFR = -21 \times \ln(\text{aortic PWV}) + 125$) and in healthy controls ($r = -0.383$, $p = 0.010$, corresponding regression line; $eGFR = -18 \times \ln(\text{aortic PWV}) + 115$) were observed.

PWV of the total aorta compared to healthy volunteers for each given eGFR ($p = 0.047$). No significant difference between groups in PWV of the proximal ($p = 0.208$) and distal ($p = 0.065$) aorta was observed for each given eGFR (**Table 2**). Interaction analyses showed that the inverse association between renal function and total aortic PWV was not significantly different between patients with hypertension and healthy volunteers ($p = 0.783$). Therefore, the decrease in eGFR per increase PWV of the total aorta did not differ between patients and healthy volunteers. The inverse association between total aortic PWV and eGFR was independent of age, diabetes mellitus, blood pressure, BMI, smoking status and hypertension duration ($p = 0.042$, $\text{Beta} = -0.279$).

DISCUSSION

The three main findings of the present cross-sectional study are 1. Patients with hypertension show increased MR assessed regional aortic stiffness of the proximal and distal aorta; 2. Changes in renal function within the normal range are inversely related to arterial stiffness even in the absence of disproportionate stiffening; 3. This relation is similar in hypertension and healthy volunteers and present before the onset of decreased eGFR, proteinuria and macrovascular complications.

Table 2. Linear regression analysis

eGFR (ml/min/1.73m ²)		A±SE	p*	B±SE	p [#]	Spearman R
PWV _{proximal}	Patients with hypertension	-14±6 (p=0.002)	-	111±10 (p<0.001)	0.208	-0.312 (p=0.002)
	Healthy volunteers	-14±5 (p=0.011)		108±10 (p<0.001)		-0.326 (p=0.031)
PWV _{distal}	Patients with hypertension	-15±5 (p=0.002)	-	114±9 (p<0.001)	0.065	-0.329 (p=0.001)
	Healthy volunteers	-15±5 (p=0.024)		109±11 (p<0.001)		-0.346 (p=0.021)
PWV _{total}	Patients with hypertension	-21±6 (p<0.001)	0.783*	125±11 (p<0.001)	0.047*	-0.380 (p<0.001)
	Healthy volunteers	-18±7 (p=0.012)		115±12 (p<0.001)		-0.383 (p=0.010)

The table illustrates the linear regression analysis $eGFR = A \times \ln(\text{aortic PWV}) + B$ for patients with hypertension and healthy volunteers. Multiple regression analysis showed an increased PWV of the total aorta in patients with hypertension compared to healthy volunteers for each given eGFR ($p^{\#}=0.047$) was found. The rate of decrease of eGFR per increase in total aortic PWV was not statistically significantly different between patients with hypertension and healthy volunteers ($p^*=0.783$)

The existing link between chronic kidney disease and arterial stiffness is a widely known phenomenon (2,12) however, so far few studies have reported on arterial stiffness and renal function in the normal range. Mourad et al. observed an independent association between PWV and kidney function in 1290 subjects with normal or elevated blood pressure (13). In line, the Hoorn study found a significant independent association between arterial stiffness measured as distensibility of the common carotid artery, and renal function (14). However, the associations in these studies were solely found in the lowest tertile of creatinine clearance or in populations including subjects with mild impaired renal function. Schillacie et al. were the first to demonstrate a relationship between aortic PWV and GFR within the normal range (serum creatinine < 120 µmol/l) in 305 treatment naive hypertensive patients (15). Compared to our study, those patients had a shorter hypertension history and were older with a mean age of 49 years. Because both renal function and arterial stiffness are dependent on age it is relevant to determine whether this relation still exists in younger subjects. Furthermore, all previous studies used echo Doppler or applanation tonometry to determine global arterial wall stiffness of the aorta. These modalities are limited by the inability to register the true length of the aorta and need geometric assumptions to estimate PWV. MR as imaging modality has been validated and applied to study regional aortic PWV and with this technique we are the first to describe a relation between renal function within the normal range and regional aortic PWV in hypertensive patients. In contrast to others, our results were not

confounded by kidney disease as we selected a population with normal renal function and absence of proteinuria.

Our findings stress the feasibility of velocity-encoded MRI for evaluation of regional aortic stiffness. Intriguingly, although increased PWV was present for both segments of the aorta, we did not find increased proximal compared to distal aortic stiffening in patients with hypertension. Because central stiffening is believed to occur mainly beyond the fifth decade (16) this may be attributed to our young study population (only 17% older than 60 years). In addition, PWV values were only mildly elevated compared to healthy volunteers and appeared to be within the range of reported values from intra-arterial pressure measurements which is regarded as the gold standard. With this technique PWV increased from 4-5m/s in the ascending aorta to 5-6m/s in the abdominal aorta (17) in subjects without hypertensive disease (mean age 42 years).

Disproportionate aortic stiffening in aging and hypertension may promote transmission of pulsatile energy towards the microcirculation and expose end organs to potential damaging effects of high pulse pressure. Possibly the absence of renal damage in our subjects might be explained by the absence of disproportionate stiffening rather than stiffening per se. In addition, it is conceivable that the kidney is adapting in response to arterial stiffness and increased pulse pressure. Renal function may then be preserved at the cost of microvascular remodeling or altered activation of the renin angiotensin-aldosterone system. In support of this hypothesis, studies in community-based samples have demonstrated altered microvascular structure and function in the presence of aortic stiffness (18). Recently in an elderly population cohort, the association between PWV and GFR was mediated in part by higher renal artery pulsatility index and higher renal vascular resistance (6). On the other hand, microvascular renal damage is not necessarily reflected by decreased GFR. By example, microalbuminuria provides additional information next to GFR values and is believed to reflect endothelial dysfunction. Moreover, it is well known that any formula for estimating GFR has a low precision for assessing GFR and tends to be less accurate in subjects with relatively normal renal function (19). MR assessed PWV may then be a possible early marker of microvascular renal damage which is not yet detected by GFR.

Our study has some limitations. First, the inclusion of patients treated with various drugs might have affected the results. Angiotensin converting inhibitors and blockers of the renin-angiotensin system in particular, may lower PWV (8,20,21) and slow onset of kidney disease. Although appropriate statistical analysis was used to adjust for medication effects, this may lead to an underestimation of the association between PWV and GFR and the true association is likely to be even stronger than observed. In addition, because of the cross-sectional nature of this study we cannot determine the changes over time

and further studies are needed to establish whether increased PWV is a marker of future decline in renal function.

In conclusion, the present study shows that changes in renal function within the normal range are inversely related to increased arterial stiffness even in the absence of disproportional stiffening. This relationship is similar in patients with hypertension and healthy volunteers and present before the onset of decreased eGFR, proteinuria and macrovascular complications. These results stress the close link between aortic stiffness and the microcirculation. Future studies are needed to elucidate the implications of early arterial stiffening in hypertension on long-term renal function.

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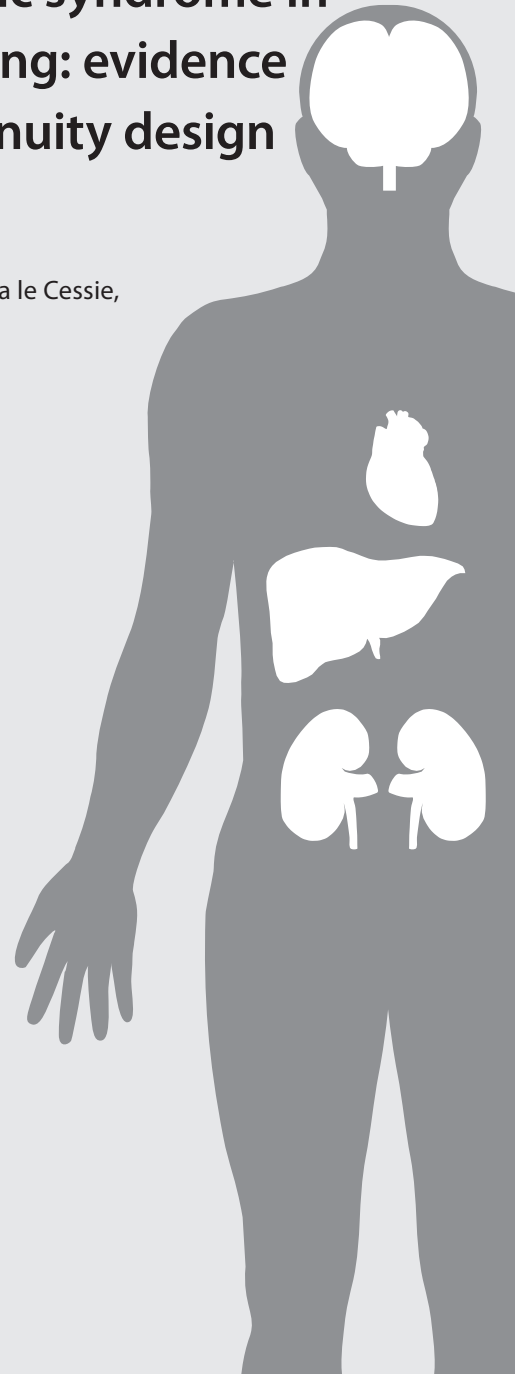
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Chapter 8

Impact of a lifestyle intervention among subjects with the metabolic syndrome in a primary health care setting: evidence from a regression discontinuity design

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Submitted



ABSTRACT

Background

The metabolic syndrome (MetS) is a well-known entity related to cardiovascular disease and type 2 diabetes. Many subjects with MetS are affected by central obesity. Randomized clinical trials have demonstrated that lifestyle changes exert beneficial effects on obesity and other MetS-components. However these trials have been performed under highly controlled conditions and the generalizability across persons, settings and outcomes may be limited.

Aim

The purpose of this study was to evaluate the feasibility and the effect of the implementation of a lifestyle intervention (inclusive of possible blood pressure treatment) in subjects with the MetS in a primary health care setting. The effect was assessed using a regression discontinuity design.

Methods

In four primary health care centers 2079 of all 2942 invited non-diabetic subjects between 40 and 70 years of age (response rate = 71%) responded to their general physician's request for screening. Subjects with the MetS but without diabetes ($n=686$) were identified from the total group, using a screening protocol. Insulin resistance ($\text{HOMA-IR} \geq 2$) was used as defining factor for lifestyle intervention. A regression discontinuity design was used to estimate the effect of the lifestyle intervention. Subjects with the MetS and $\text{HOMA-IR} \geq 2$ were offered a lifestyle program. Subjects with MetS and $\text{HOMA-IR} < 2$, the non-intervention group, received a letter containing a set of lifestyle advices (amongst which also possible referral for blood pressure treatment). In the regression discontinuity design posttest scores were regressed on pretest scores with group as a covariate. For the effect on visceral obesity, glucose, lipid levels and blood pressure HOMA-IR was added as a second covariate.

Results

Seventy-eight subjects were included in the lifestyle intervention program and 311 subjects were included in the non-intervention group. The intervention reduced visceral obesity [waist circumference -2.99 cm (95% CI $-4.72, -1.26$)] and lowered systolic blood pressure [-7.28 mmHg (95% CI $-12.78, -1.79$)] compared to the non-intervention group. This blood pressure lowering effect was also present amongst the persons who did not receive any new blood pressure lowering medication. Insulin resistance did not significantly change in subjects whom received lifestyle intervention [adjusted for pre- HOMA-IR values the intervention group had a 1.17 times (95%CI 0.99-1.39) higher

post-HOMA-IR. Furthermore no significant change in subjects with lifestyle intervention compared to the non-intervention group was found for diastolic blood pressure [1.85 mmHg (95% CI -4.96, 1.25) lower], glucose [0.06 mmol/l (95% CI -0.23, 0.12) lower], HDL-cholesterol [0.99 (95% CI 0.94, 1.03) times lower and triglycerides levels [0.99 mmol/l (95% CI 1.03 (95% CI 0.89, 1.12) times lower].

Conclusions

A lifestyle and dietary intervention, with the added possibility of blood pressure treatment, can be implemented in a primary care setting using existing facilities and was found to effectively reduce visceral obesity and lower systolic blood pressure in MetS-subjects. Therefore this study may provide an effective strategy for clinical health systems to meet the growing challenge of obesity and cardiovascular disease.

INTRODUCTION

The World Health Organization estimates that more than 1 billion adults worldwide are overweight (1). Excess weight, particularly abdominal or central obesity is related to the metabolic syndrome (MetS). The MetS increases the risk to develop cardiovascular disease and type 2 diabetes mellitus. Two large meta-analyses have shown that the risk for cardiovascular disease (CVD) is increased approximately 2 fold in MetS (2;3). As central obesity is a key factor in the development of insulin resistance and MetS, appropriate management of central obesity is regarded as an important initial step to reduce the burden of CVD.

Randomized clinical trials (RCT's) have demonstrated that lifestyle interventions, including dietary modification and increased physical activity, exert beneficial effects on each of the individual components of the MetS (4-6).

RCT's are regarded as the 'gold standard' for clinical research designs. Still, RCT's are limited in their generalizability across persons, settings, treatments and outcomes. Most clinical trials have been performed with highly selected participants under highly controlled conditions which is different from the situation of daily practice. Therefore the effectiveness of measures taken in everyday practice with relatively unselected participants under flexible conditions may be different from the results obtained using RCTs.

In a primary care setting performing a RCT is not always possible, especially when evaluating the efficacy of an intervention in a "real" world setting is the objective (7). The regression discontinuity design is the most suitable alternative when randomization is not feasible or acceptable. In the regression discontinuity design, assignment of individual subjects or groups to intervention is determined by the use of a "cut-off" score measured at baseline. Through regression adjustment for the assignment score an unbiased estimate of the intervention effect can be obtained.

The purpose of this study was to evaluate the feasibility and the effect of the implementation of a lifestyle intervention in non-diabetic MetS-subjects in primary health care setting. The regression discontinuity design was used to estimate the effect of the intervention.

MATERIALS AND METHODS

Study subjects

This prospective study was conducted between February 2006 and August 2010, in four primary health care centers in Rijswijk, the Netherlands. The study protocol was approved by the review board of South West Holland and performed in accordance

with the Declaration of Helsinki. During 2006-2008 all non-diabetic subjects between 40-70 years of age of four general practitioners were identified using computer assisted screening. Exclusion criteria were diabetes mellitus, unstable medical condition, major psychiatric comorbidity or a history of drug or alcohol abuse. A total of 2942 subjects were considered eligible and invited to the practices by letter. Invitation letters were sent in order of month of birth. A second letter was sent for non-responding subjects. Screening was carried out on 2079 subjects (response rate = 71%) after written informed consent. Subjects with the MetS (for criteria see below) were selected and invited to participate in this study. Subjects with the MetS with low levels of insulin resistance ($\text{HOMA-IR} < 2$) received health care advice by letter. This is not strictly a control group as used in randomized clinical trial and therefore this group will be referred to as the non-intervention group in the remainder of this manuscript. Subjects with the MetS with high levels of insulin resistance ($\text{HOMA-IR} \geq 2$) received lifestyle intervention. Subjects with new diagnosed type 2 diabetes defined by a glucose ≥ 7 mmol/l at screening were excluded for both arms of the study. All subjects gave informed consent.

Measurements and definitions

During the screening visit, medical history including use of medication was taken, if necessary additional information was retrieved from the medical record. Subsequently, waist circumference was measured in standing position, midway between the lower limit of the rib cage and the iliac crest using a Seca 200 circumference measuring tape (Seca GmbH, Hamburg, Germany). Brachial arterial blood pressure was measured twice in the left arm with an automatic digital BP monitor (Omron M7, Hoofddorp, The Netherlands) while subjects were relaxed in a seating position for at least five minutes. The average of the two readings was used to calculate systolic blood pressure and diastolic blood pressure. A third measurement was taken if the difference between the two readings exceeded 10 mmHg and the average of the second and third measurement was used. Venous blood samples were taken after a 12-h overnight fast and included glucose, insulin, triglycerides, and HDL-cholesterol levels. Blood samples were analyzed at the central laboratory facilities of the general physicians at Rijswijk in collaboration with the department of Clinical Chemistry at Reinier de Graaf Hospital in Delft, The Netherlands. Measurements were performed according to ISO 15189 standard procedures. Glucose levels were determined by colorimetric enzymatic methods on an Architect c8000 analyzer. Insulin was measured using an immunometric chemoluminescent immunoassay (Immulite 2000).

The MetS was defined using the IDF criteria (8). Therefore two or more of the following criteria in addition to an increased waist circumference (male ≥ 94 cm, female ≥ 80 cm), had to be present: triglycerides ≥ 1.7 mmol/l (≥ 150 mg/dl), HDL-cholesterol < 1.03 mmol/l (< 40 mg/dl) in male and < 1.29 mmol/l (< 50 mg/dl) in female, fasting glucose

≥ 5.6 mmol/l (150 mg/dl), blood pressure $\geq 130/85$ mmHg or the use of blood pressure lowering medication. Insulin resistance was calculated using the Homeostasis Model Assessment-Insulin Resistance Model (HOMA-IR), according to the published algorithm (9).

Health care advice by letter

After the screening the non-intervention group i.e. MetS-subjects with low levels of insulin resistance (HOMA-IR < 2) received a letter with information regarding their cardiovascular risk and a set of lifestyle advices about general health. Recommendations regarding nutrition (i.e. to eat daily 200 gram vegetables and two pieces of fruit, to eat fish at least twice a week and to consume little or no added salt) and exercise (i.e. to engage in physical activity of moderate intensity for at least 30 minutes 5 times a week) were given. If applicable quit-smoking advice was given and subjects with hypertension (blood pressure $\geq 140/90$ mmHg) or dyslipidemia in combination with a high cardiovascular risk (10) were advised to visit their general practitioner for further treatment. These subjects were treated according national guidelines regarding cardiovascular risk.

Lifestyle intervention

The intervention consisted of a lifestyle program and was adapted on the step one approach of type 2 diabetes mellitus treatment in the Netherlands (11). In this approach an individual lifestyle and dietary intervention counseling is given to subjects according to these guidelines. A nurse practitioner with 3 years of experience in diabetes counseling provided four sessions; immediately after screening and after three, six and nine months.

The goals of the intervention program were to achieve and maintain a weight reduction of at least 5% of initial body weight through consumption of a healthy low calorie, low fat diet and to engage in physical activity of moderate intensity for at least 30 minutes 5 times a week. Counseling consisted of planning, motivation and explanation of activating aspects of the registered lifestyle patterns including exercise and smoking. Dietary counseling emphasized the content fibers, fish and vegetable intake. An upper limit of calorie intake of 2100 kCal was advised. If weight loss was not observed after three months, special emphasis was given aiming at a reduction in weight of at least 3-5%. As for the non-intervention group, medical treatment prescribed by a primary physician was allowed according to national guidelines for hypertension, cardiovascular prevention and diabetes. Prescription of glucose lowering medication was not permitted during the program. Weight and blood pressure were measured at each visit. Subjects with a high blood pressure ($\geq 140/90$) were treated according to guidelines (10).

Follow up measurements

A fasting blood sample was drawn for measurement of glucose, insulin, triglycerides, and HDL-cholesterol levels after the last visit to the nurse practitioners in subjects in the intervention group. In subjects of the non-intervention group a fasting blood sample and measurements of blood pressure and waist circumference were repeated nine months after subjects received health care advice by letter.

Study Outcomes

The predefined outcomes were the change in 9 months HOMA-IR, visceral obesity, glucose, cholesterol levels and blood pressure.

Statistical analysis

Data are expressed as mean $\pm$ standard deviation or median (interquartile range), unless stated otherwise. To compare clinical characteristics between the intervention and the non-intervention group at baseline independent samples t-test for continuous variables and a Mann-Whitney for non-parametric continues variables were used. For the comparison between dichotomous variables a Chi-Square test was performed. A paired t-test or Wilcoxon-matched-paired test was used to compare outcomes before and after the intervention. Because HDL-cholesterol, triglycerides and HOMA-IR were non-normally distributed a log transformation of these values was used in regression analysis. A P value < 0.05 was considered statistically significant. We used SPSS for Windows (version 18.0; SPSS, Chicago, Illinois, USA) for statistical analysis.

Comparing pre- and post-measurements in the intervention group is hampered by regression to the mean, because the intervention group was selected based on high baseline HOMA-IR values. Therefore we also performed a regression discontinuity design, in which assignment of subjects to (lifestyle) intervention is solely determined by the use of a “cut-off” score measured at baseline. The intervention effect is not indicated by a difference between the posttest averages of groups but rather by a change in the pre-post relationship at the cutoff point. Through regression adjustment for the assignment score an unbiased estimate of the intervention effect can be obtained. Treatment effects in regression discontinuity design are estimated by fitting least squares regression lines regressing posttest scores on pretest scores with group as a covariate and if needed, an interaction term between group and pretest score (12).

Several possible results of regression discontinuity analyses are illustrated in **Figure 1**. All subjects are assigned to treatment or standard care based on the pre-test cut-off score of 2 in this fictitious situation. The first graph in **Figure 1** illustrates a case in which there is no treatment effect (model a). This is indicated by the regression line that runs straight through the data-points on both sides of the cut-off. The second graph (model

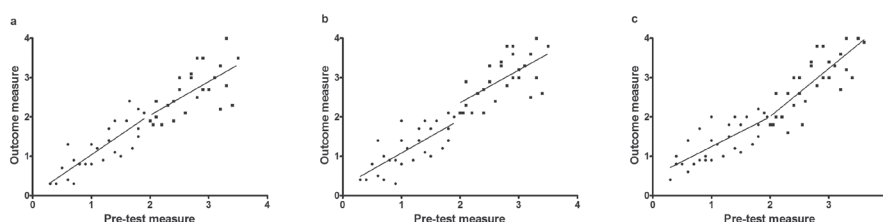


Figure 1. Possible results of regression discontinuity. Model a illustrates a case in which there is no treatment effect i.e. the regression line runs straight through the data-points. Model b shows the classic regression discontinuity design. The treatment exerts a positive effect, as expressed by the 'discontinuity' of the regression line at the cut-off. Model c demonstrates an additional slope of the regression line; a model in which interaction was allowed.

b) shows the classic regression discontinuity design. The treatment exerts a positive effect, as expressed by the 'discontinuity' of the regression line at the cut-off point. The third graph in **Figure 1** demonstrates a regression discontinuity with an additional slope of the regression line; in the models a and b, the regression lines below and above the cut-off point have identical slopes but in model c interaction is allowed.

Using the regression discontinuity design we analyzed the following outcomes: a) change in the 9-month HOMA-IR, glucose, waist circumference, blood pressure, the level of triglycerides and HDL-cholesterol. Therefore we regressed the 9-month HOMA-IR on baseline HOMA-IR with group as a second covariate. The regression coefficient for group then reflects the effect of the intervention for patients with the cut-off value of HOMA-IR. For the effect on 9-month glucose, waist circumference, blood pressure, the level of triglycerides and HDL-cholesterol the assignment score (baseline HOMA-IR) was added as a second covariate. Interaction terms for pre-tests were added if they significantly improved the R^2 for the 9-month outcome. Regression coefficients for log transformed variables were exponentiated, in this way they reflect how much larger the (geometric) mean in the intervention group is compared to the non-intervention group.

RESULTS

Study participants

From all eligible and non-diabetic subjects aged 40-70 years, 2079 (71%) participated in the screening phase of the study. The flow scheme of the inclusion is shown in **Figure 2**. Six hundred eighty-six subjects were diagnosed with the MetS and invited to participate in the second part of the study. Subjects with high insulin resistance levels (HOMA-IR ≥ 2 , $n=144$) were assigned to a 9 months lifestyle intervention program during the period

February 2006 to August 2010. Of the seventy-eight subjects willing to participate, nine subjects withdrew informed consent and sixty-nine subjects followed the program. Subject willing to participate ($n=78$) did not differ from subjects not included in the intervention group ($n=66$) with respect to baseline characteristics, besides from a higher percentage of subjects with high triglycerides (65% vs 50%, $p=0.049$). Subjects with insulin resistance levels below the cut-off were assigned to the non-intervention group ($\text{HOMA-IR} < 2$, $n=543$). In this group 311 subjects were willing to participate and received lifestyle advice by letter. Subjects with insulin resistance below the cut-off and willing to participate were older (56.1 vs 53.9 years, $p=0.030$) and less likely to be diagnosed with impaired fasting glucose compared to the subjects not included in the non-intervention group (40% vs 73%, $p=0.001$). Baseline characteristics of both groups are listed in **Table 1**. Overall the mean age was 56 ± 8 years. Median HOMA-IR was 2.50 (2.30 - 3.20) in the intervention and 1.20 (0.80 - 1.50) in the non-intervention group. Groups were comparable with respect to age, gender, smoking, family history of cardiovascular disease and use of lipid lowering medication. By selection the metabolic syndrome was present in all subjects. Subjects in the intervention group more often fulfilled the criteria for low HDL-cholesterol (72% vs 59%, $p=0.045$) and elevated levels of triglycerides (65% vs 50%, $p=0.012$). Furthermore more subjects used blood pressure lowering medication (50% vs 34%, $p=0.019$) in the intervention group compared to the non-intervention group.

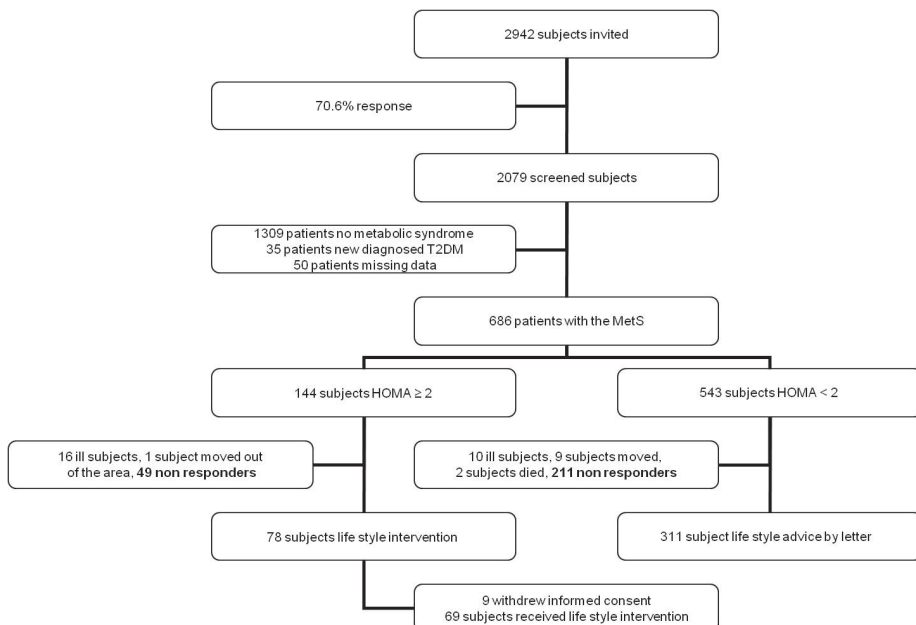


Figure 2. Flowchart of enrollment and assignment of subjects based on a cut-off score for insulin resistance ($\text{HOMA-IR} \geq 2$). Subjects above the cut-off score were assigned to a lifestyle intervention and subjects below the cut-off score were assigned to the non-intervention group.

Table 1. Clinical characteristics of the lifestyle intervention group and the non-intervention group at baseline.

	Intervention group N=78	Non-intervention group N=311	P
Age (years)	55.7 (7.9)	56.1 (8.2)	0.717
Male gender, n (%)	40 (51)	176 (57)	0.399
Smoking status, n (%)	27 (35)	92 (30)	0.388
Family history of cardiovascular disease, n (%)	23 (29)	71 (23)	0.382
Lipid lowering medication, n (%)	18 (23)	60 (19)	0.455
Blood pressure lowering medication, n (%)	38 (50)	107 (34)	0.019*
Metabolic syndrome defining criteria			
Central obesity, n (%)	78 (100)	311 (100)	-
High blood pressure [#] , n (%)	69 (88)	266 (86)	0.503
Low HDL-cholesterol [#] , n (%)	56 (72)	185 (59)	0.045*
High triglycerides [#] , n (%)	51 (65)	154 (50)	0.012*
Impaired fasting glucose [#] , n (%)	50 (64)	169 (54)	0.120

Abbreviations; HDL: high density lipoprotein. Values represent mean $\pm$ standard deviations or number of subjects (percentages). *P < 0.05. [#]IDF defined MetS criteria: central obesity waist circumference male $\geq$ 94 cm and female $\geq$ 80 cm, HDL-cholesterol male < 1.03 mmol/l and female < 1.29 mmol/l, triglycerides > 1.7 mmol/l, impaired fasting glucose $\geq$ 5.6 mmol/l, blood pressure; systolic blood pressure $\geq$ 130 mmol/l and/or diastolic blood pressure $\geq$ 85mmol/l and/or treatment with blood lowering medication.

Measurements at follow up

The change in metabolic syndrome components and insulin resistance (HOMA-IR) after nine months of the intervention group and non-intervention group are shown in **Table 2** and **3**. The subjects following the intervention protocol visited the nurse practitioner on average 3.5 ± 1.3 times during the 9 months treatment period. Nine subjects withdrew informed consent before the start of the lifestyle program and 4 subjects did not complete all four sessions. These subjects were analyzed according to the intention-to-treat principle. During the nine months follow-up, five (6.4%) subjects of the intervention group and twelve (3.9%) subjects of the non-intervention group, developed type 2 diabetes (defined as a fasting glucose > 7 mmol/l). A new prescription of antihypertensive medication, an increase in the number of antihypertensive drugs or a rise in dose was observed in 18 (23.1%) subjects of the intervention group and in 42 (13.5%) subjects of the non-intervention group. Statins were prescribed in five (6.4%) subjects during lifestyle intervention and in six subjects (1.9%) of the non-intervention group.

Results of the regression discontinuity design analysis

Results are presented from linear models without interaction terms for pretests, as interaction terms did not improve the model significantly in any of the situations. A decline in waist circumference [-2.99 cm (95% CI -4.72, -1.26)] and a reduction in systolic blood

Table 2. Metabolic syndrome components and insulin resistance (HOMA-IR) of the intervention group and non-intervention group at baseline and after 9 months

	Intervention group N=78			Non-intervention group N=311		
	Baseline	After 9 months	P	Baseline	After 9 months	P
Waist circumference, cm						
- Male	109 ± 8	106 ± 8	<0.001*	103 ± 8	103 ± 8	0.806
- Female	104 ± 16	102 ± 16	0.035*	96 ± 9	97 ± 10	0.235
HDL-cholesterol, mmol/l						
- Male	0.91 (0.81 - 1.10)	0.95 (0.81 - 1.06)	0.901	1.00 (0.88 - 1.18)	1.01 (0.89 - 1.14)	0.964
- Female	1.13 (0.98 - 1.28)	1.10 (0.97 - 1.23)	0.186	1.23 (1.06 - 1.53)	1.27 (1.10 - 1.48)	0.596
Triglycerides, mmol/l	1.95 (1.53 - 2.77)	2.00 (1.33 - 2.64)	0.823	1.69 (1.12 - 2.30)	1.74 (1.25 - 2.29)	0.053
Glucose, mmol/l	5.70 ± 0.63	5.84 ± 0.65	0.026*	5.50 ± 0.65	5.71 ± 0.64	<0.001*
Systolic blood pressure, mmHg	144 ± 23	138 ± 19	0.009*	142 ± 19	142 ± 18	0.819
Diastolic blood pressure, mmHg	89 ± 11	85 ± 10	<0.001*	87 ± 10	86 ± 11	0.026*
HOMA-IR	2.50 (2.30 - 3.20)	2.30 (1.60 - 2.90)	0.002*	1.20 (0.80 - 1.50)	1.10 (0.80 - 1.70)	0.033*

Abbreviation: HDL: high density lipoprotein, HOMA-IR: Homeostasis Model of Assessment – Insulin Resistance. Values represent mean ± standard deviation or medians (interquartile range). *P<0.05 vs baseline.

Table 3. Change in metabolic syndrome components and insulin resistance (HOMA-IR) of the intervention group and non-intervention group

	Change in the intervention group	Change in the non-intervention group	P
Waist circumference, cm			
- Male	-3 ± 4	0.1 ± 4	<0.001*
- Female	-3 ± 7	0.7 ± 6	0.010*
HDL-cholesterol, mmol/l			
- Male	0.02 (-0.11 - 0.10)	0.00 (-0.09 - 0.10)	0.949
- Female	-0.04 (-0.04 - 0.10)	0.10 (-0.11 - 0.15)	0.488
Triglycerides, mmol/l	0.00 (-0.42 - 0.32)	0.10 (-0.43 - 0.32)	0.521
Fasting blood glucose, mmol/l	0.15 ± 0.57	0.21 ± 0.60	0.063
Systolic blood pressure, mmHg	-5.8 ± 18.9	-0.3 ± 18.7	0.027*
Diastolic blood pressure, mmHg	-4.2 ± 9.4	-1.4 ± 10.5	0.034*
HOMA-IR	-0.50 (-0.30 - 1.00)	0.00 (-0.50 - 0.30)	<0.001*

Abbreviations; HDL: high density lipoprotein, HOMA-IR: Homeostasis Model of Assessment – Insulin Resistance. Values represent mean ± standard deviation or medians (interquartile range) of the change during 9months follow up. *P<0.05.

pressure [-7.28 mmHg (95% CI -12.78, -1.79)] in the intervention group compared to the non-intervention group was found (**Table 4, Figure 3-5**). Looking at the subgroup of subjects without antihypertensive agents or in whom medication was not increased (76% of the intervention group and 86% of the non-intervention group 86%) similar results for systolic blood pressure were observed [-7.72 mmHg (95% CI -13.07, -2.36)]. Insulin resistance [1.170 units (95% CI 0.985, 1.388)] HOMA-IR higher in the intervention group, **Figure 5**) did not significantly change compared to the non-intervention group. Similarly, no significant change compared to the non-intervention group was found for diastolic blood pressure [1.85 mmHg (95% CI -4.96, 1.25) lower], glucose [0.06 mmol/l (95% CI -0.23, 0.12) lower], HDL-cholesterol [0.99 (95% CI 0.94, 1.03) times lower] and triglycerides [0.99 mmol/l (95% CI 0.89, 1.11) times lower].

DISCUSSION

This study shows that lifestyle intervention can be successfully implemented in a primary health care center by nurse practitioners using the step one approach as described in the national dutch diabetes protocol. Thus showing the feasibility of lifestyle intervention in primary care. Furthermore, the intervention successfully reduced visceral obesity and lowered systolic blood pressure. Despite a reduction in obesity the intervention did not significantly affect insulin resistance levels in individuals with the metabolic syndrome.

Table 4. Results of regression discontinuity design analysis

Covariates	Intervention effect B (95% CI)	P
LN HOMA-IR	0.157(-0.015, 0.328)	0.074
HOMA-IR, units [#]	1.170 (0.985, 1.388) times higher compared to the non-intervention group	
Waist circumference, cm	- 2.99 (-4.72, -1.26)	0.001*
LN HDL-cholesterol	- 0.01 (-0.06, -0.03)	0.546
HDL-cholesterol, mmol/l [#]	0.99 (0.94, 1.03) times lower compared to the non-intervention group	
LN triglycerides	-0.01 (-0.12, 0.11)	0.910
Triglycerides, mmol/l [#]	0.99 (0.89, 1.11) times higher compared to the non-intervention group	
Fasting blood glucose, mmol/l	-0.06 (-0.23, 0.12)	0.542
Systolic blood pressure, mmHg	-7.28 (-12.78, -1.79)	0.010*
Diastolic blood pressure, mmHg	-1.85 (-4.96, 1.25)	0.240

Abbreviations; HDL: high density lipoprotein, HOMA-IR: Homeostasis Model of Assessment – Insulin Resistance. Values represent regression coefficients (95% confidence intervals) for the intervention group in a regression model with as independent variable the posttest and the pretest, group and assignment score (HOMA-IR) as covariates. [#]Exponentiated regression coefficient of the model with the log transformed outcome. *P<0.05

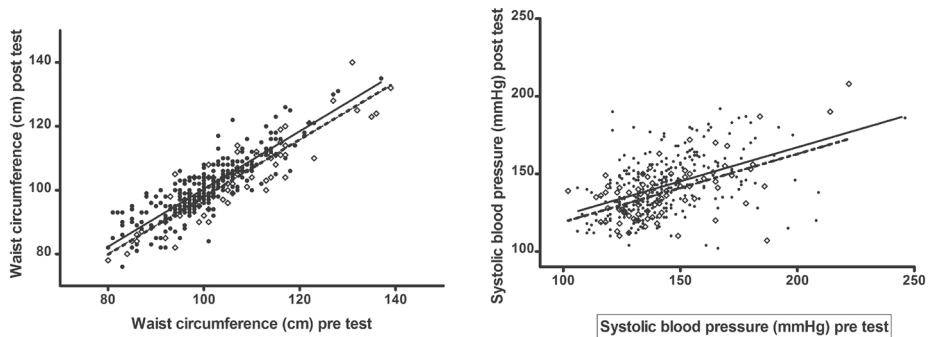


Figure 3 (left panel) and **4** (right panel). Regression discontinuity analysis regarding the effect on waist circumference (Figure 3) and systolic blood pressure (Figure 4). Posttest scores were regressed on pretest scores with group and HOMA-IR score as additional covariates and predicted regression lines for both groups separately are shown. Waist circumference declined with -2.99 cm (95% CI -4.72, -1.26) in the intervention group compared to the non-intervention group. Systolic blood pressure was reduced with -7.28 mmHg (95% CI -12.78, -1.79) in the intervention group compared to the non-intervention group. Legend: • = non-intervention group, ---- = life style intervention group

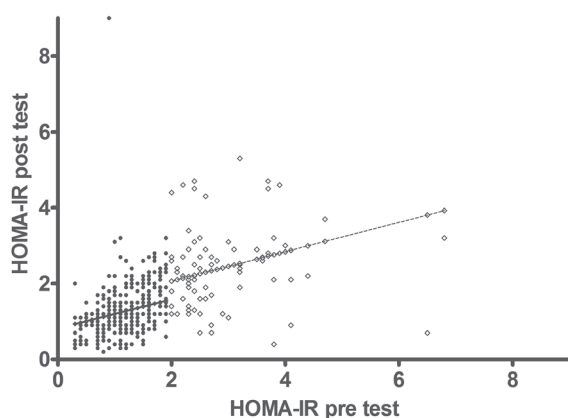


Figure 5. Regression discontinuity analysis regarding the effect on insulin resistance measured as HOMA-IR; posttest scores were regressed on pretest scores with group and assignment score as a second and third covariate and predicted regression lines for both groups separately are shown. HOMA-IR was 1.170 (95% CI 0.985, 1.388) higher in the intervention group compared to the non-intervention group. Legend: $\cdot$ = non-intervention group, $----$ = lifestyle intervention group.

The MetS is relatively widespread in Western populations and is of considerable public health concern (1). In line, we observed MetS in 686 (33%) non-diabetic subjects in our study. International guidelines recommend lifestyle as a first line therapeutic strategy for reducing the risk of diabetes and cardiovascular disease (13). With growing number of individuals with obesity and MetS implementation of lifestyle intervention becomes a major public health care challenge. Lifestyle interventions that can be implemented in existing community health care systems may be advantageous in terms of time needed for implementation and health care budgets. This study underlines these possibilities by showing the feasibility of lifestyle intervention therapy in primary health care centers.

The lifestyle intervention was successful in reducing visceral obesity and lowering blood pressure. The blood pressure lowering effect in the intervention group compared to the non-intervention group could not be solely attributed to drug treatment as the estimated reduction in blood pressure was similar in subjects without antihypertensive medication. Our results are in line with previous studies showing a beneficial effect on each of the components of the MetS (4-6).

In contrast to these randomized controlled trials we observed relatively modest reductions in waist circumference [-2.99 (-4.72, -1.26) cm]. Furthermore we did not observe an effect for triglycerides, glucose and insulin resistance levels. A possible explanation is that a relative low intensity of intervention was used. Moreover our estimate of the intervention effect can be considered "conservative" since the data were analyzed according to the intervention-to-treat principle. Additionally all subjects assigned to the non-intervention group also received general health advice by letter at base line and may have benefited from the advice. On the other hand HOMA-IR is a relatively crude method for the quantification of insulin resistance. HOMA-IR is based on the product of fasting insulin and glucose and only a basal measurement is used. Fluctuation in the

basal overnight fasting insulin concentration is a major source of this variability which may explain why we did not find an effect for the relative small intervention group.

Few studies on lifestyle modification in metabolic at risk subjects have been performed in the primary health care setting. In contrast with our results, the CELL Study Group found no effect of an intensive health care advice given by a general practitioner and nurses working in primary care. This study was mainly carried out in male with hyperlipidemia but did not necessarily include subjects with the MetS (14). In the setting of a rural primary practice in Germany a weight management program with two weekly training sessions was found to improve all metabolic abnormalities and blood pressure in subjects with the MetS after one year (15). Compared to our results larger reductions in obesity and blood pressure were observed. However the beneficial effect might be overestimated because the assignment to the treatment groups was not randomized. In a clinical research setting the DASH diet has been shown to reduce weight (16 kg) and to lower blood pressure (12 mmHg) in the MetS (4). Two other RCT's carried out in subjects with the MetS demonstrated reductions in waist and blood pressure with a diet or exercise programs (16;17). The difference in effect of our intervention with respect to that obtained in these trials (4;16) can be explained by differences in methods and intensity of interventions applied. Trials were performed in clinical outpatient clinics from university centers, subjects were often self-referred, more obese and weight loss is obtained during a longer intensive program more difficult to implement in daily practice.

Our findings may be more generalizable to daily practice in primary health center settings than are those of highly controlled randomized trials with larger treatment effects. The successful adaptation of the intervention from the diabetes protocol and implementation using the preexisting structure of diabetes counseling by nurse practitioners are highly encouraging to consider wider application of our approach. In addition the employment of nurse practitioners provides the advantage of an established link between the patient and physician and experience already exists in management of co-morbidity.

Several considerations affect interpretations drawn from our findings. First, we did not use a randomized clinical trial design. Second, when using existing primary health care facilities, limitations were encountered with the number of counseling sessions. Third, the study sample size was relatively small and patient engagement with the intervention was less than desired but not inconsistent with other trials. Fourth, our intervention results apply primarily to MetS-subjects. Whether the results would be similar in obese persons without the MetS is likely but unknown. Furthermore, the period of evaluation was relatively short and this study cannot provide information about long-term efficacy and effectiveness.

The regression discontinuity design is a more appropriate approach than the often used pre-post design with no control group and has been proposed as a suitable technique for use in the public health domain (12).

However several assumptions must be met before using the regression discontinuity design (12, 18). The first assumption is that treatment assignment around the cut-off point is as good as random, otherwise selection bias may be induced. As subjects in our study were not able to manipulate their HOMA-IR and thereby their assignment to the intervention or non-intervention group this assumption is likely to be met. Second, it is important to correctly identify the relationship between assignment measure and outcome variable, hereto a sensitivity analysis with a more narrow interval around the cut-off point can be considered. The third requirement is a large enough pre-test sample size to support the use of regression analysis to estimate the pre- and posttest relationship. Fourth, all individuals in the study must be drawn from the same population. In our opinion, except for the sample size that ideally would have been larger, these criteria have been met – amongst others because we selected subjects from a population based cohort of individuals living in Rijswijk, the Netherlands. Finally it is assumed that all participants in the treatment receive the same level and amount of intervention. During the intervention not all subjects (31%) followed the complete counseling program, which reflects the real life setting.

In summary, our study shows the feasibility and success of lifestyle intervention for MetS subjects in primary health care centers. The prevention of progression of visceral obesity and MetS using such programs in primary care may provide an effective and efficient strategy for clinical health systems to meet the growing challenge of obesity and cardiovascular disease prevention.

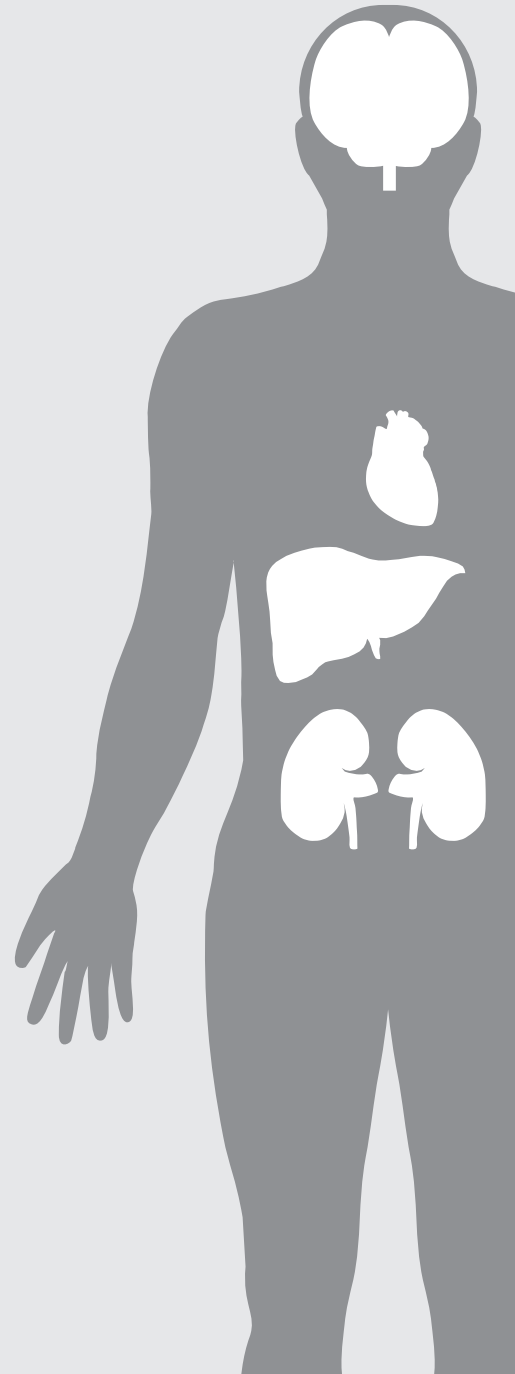
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Chapter 9

Summary and conclusions



The focus of this thesis was to evaluate biomarkers of cardiovascular end organ damage in the metabolic syndrome and diabetes mellitus. Biomarkers are characteristics that are objectively measured and evaluated and may indicate underlying disease. We performed cross-sectional studies with biochemical and magnetic resonance imaging (MRI) techniques. In addition we studied the effect of a lifestyle intervention in a general population using primary health care setting.

Chapter 1 provided a general introduction on the complex and interconnected underlying mechanisms for cardiovascular disease (CVD) and end organ damage. The relations between obesity related insulin resistance, dyslipidaemia, hypertension and inflammation were described.

Chapter 2 is a systematic review and meta-analysis on the association between fasting glucose, fasting insulin, the Homeostasis Model Assessment of Insulin Resistance (HOMA-IR) and incident CVD in individuals without diabetes. We performed the first meta-analysis that directly compared these markers with incident CVD. We included 65 cohort studies and (nested) case-control studies involving 516,325 participants and used fixed and random-effect meta-analyses to calculate the pooled relative risk for coronary heart disease, stroke or combined CVD. We demonstrated that the relative risk of CVD was higher for an increase of one standard deviation in HOMA-IR compared to an increase of one standard deviation in fasting glucose or fasting insulin concentration. Since insulin resistance may only lead to hyperglycemia when pancreatic beta cells are no longer capable to compensate by increasing insulin production we suggest that it may be useful to add markers of insulin resistance to a cardiovascular risk prediction model. Insulin resistance plays an important role in the early and advanced stages of atherosclerosis, whereas hyperglycaemia seems exclusively to be involved in early stages of atherosclerosis (1). In addition, insulin resistance seems to modify the effect of insulin on the vascular wall; anti-atherogenic in the insulin sensitive state and pro-atherogenic in the insulin resistant state. It was shown before that the addition of a fasting glucose measurement to the Framingham risk score (cardiovascular risk prediction model) resulted in a slight net reclassification improvement of 1.8% (2). Whether the addition of HOMA-IR to a risk prediction model on top of plasma glucose levels will result in a more accurate reclassification of CVD risk is unknown and should be carefully weighted against the extra costs involved.

In **Chapter 3** the role of angiopoietin-like protein 4 (ANGPTL4) in the context of inflammation was explored. It has been more than a decade since ANGPTL4 was identified and now this protein has emerged as a central player in many aspects of energy homeostasis. It is involved in a variety of functions including angiogenesis and lipoprotein metabolism.

In this study we aimed to investigate whether systemic inflammation is a determinant for plasma ANGPTL4 levels in subjects with metabolic syndrome and type 2 diabetes mellitus (T2DM). We studied four groups of male (n=334) subjects: healthy controls, subjects with metabolic syndrome without systemic inflammation, subjects with metabolic syndrome accompanied by low grade systemic inflammation and patients with T2DM. Initially subjects were matched for waist circumference and age. Plasma levels of ANGPTL4, C-reactive protein (CRP) and metabolic parameters were determined. We observed that plasma ANGPTL4 increased with inflammatory status as measured by CRP (healthy controls < subjects with metabolic syndrome without inflammation < subjects with metabolic syndrome with low grade inflammation and subjects with T2DM). Therefore plasma ANGPTL4 levels in humans are related to systemic inflammation. Furthermore, in all groups combined, plasma ANGPTL4 showed a positive correlation with CRP. To get more insight into the causal relationship between inflammation and ANGPTL4 expression we assessed whether inflammatory stimuli (i.e. Toll-like receptor 3/4 activation) increased ANGPTL4 expression using human macrophages *in vitro*. Moreover we found that *in vitro* inflammatory stimuli increased the expression and release of ANGPTL4 by macrophages. Thus far, the expression of ANGPTL4 in numerous tissues including adipose tissue and liver was thought to be mainly regulated by peroxisome proliferator-activated receptors (PPARs). Consistent with a role of PPARs, fatty acids increase ANGPTL4 expression (3-6). However we were unable to detect a correlation between plasma FFA levels and plasma ANGPTL4 levels which is likely related to the matching on visceral obesity. The current findings provide a novel link between inflammation and ANGPTL4 expression. As CRP itself is a marker rather than a mediator of inflammation, future research is needed to investigate which intracellular cascade causes an increase in ANGPTL4 expression in humans and whether the inflammation-induced increase in plasma ANGPTL4 is causally related with dyslipidemia.

In a former study we showed that ANGPTL4 reduces foam cell formation and decreases atherosclerosis in atherosclerosis-prone APO*3-Leiden mice (7). In addition we observed a negative association of ANGPTL4 with MRI assessed VWT of the carotid artery in subjects with the metabolic syndrome and systemic inflammation .

Taken these results together, the stimulation of ANGPTL4 gene expression in macrophages is specially activated by oxLDL and as shown in chapter 3 may be activated by inflammatory stimuli. Therefore increased ANGPTL4 gene expression may be a protective feedback mechanism aimed at minimizing lipid overload 1) by directly inhibiting oxLDL uptake by macrophages and 2) by inhibiting lipid loading from triglyceride-rich lipoproteins and associated activation of macrophages. Further research on ANGPTL4, inflammation and atherosclerosis in humans is necessary to evaluate the potential of targeting ANGPTL4 as a therapeutic strategy.

Chapter 4 presented the results of a study on the short-term effect of a standardized glucose load on region-specific aortic pulse wave velocity (PWV) using MRI. PWV is a validated marker for aortic stiffness, which has become an important marker for CVD and end organ damage. MRI enables regional assessment of the PWV in multiple locations due to its accuracy in determining the true path length of the propagating wave in the aorta. We performed a randomized, placebo-controlled intervention in 16 male subjects with impaired fasting glucose. The intervention consisted of an oral glucose load at one study day and water at the other study day. PWV was measured using multi-slice two-directional in-plane velocity-encoded MRI. The main finding of this study was that a standardized glucose load decreases aortic PWV within 1 hour compared with water. Furthermore the decrease was region-specific because it was observed in the proximal but not in the distal aorta. Therefore this study demonstrates region-specific changes in arterial stiffness after a glucose load. Various vascular beds and locations may react differently to the effect of insulin and other vasoactive influences. *In vitro*, the contribution of nitric oxide dependent vasodilation is larger in central arteries than in distal microvessels (8). Consistent with variation in vascular reactivity regional variation in the elastin versus collagen ratio exists within the aorta (9-11). Therefore it is possible that the effect of postprandial hyperinsulinemia would have less impact on the distal segment. Interestingly inter-individual differences in response to the glucose load were found. Subjects who did not show a significant change in PWV after the glucose load were significantly more obese. Previously an impaired insulin-induced decrease in arterial stiffness was shown in human obesity (12) and impaired nitric oxide synthesis has been reported in conditions as the metabolic syndrome (13). Future research is needed to improve knowledge on possible regional effects of hyperglycaemia on aortic stiffness and whether they link to inter-individual variation in regional vascular response.

In **Chapter 5** the impact of type 1 diabetes mellitus (T1DM) on regional grey matter volume of the brain was investigated using voxel-based morphometry (VBM). This relatively novel and sophisticated analysis technique can be used to test for regional subtle grey matter alterations. In this study 62 patients with T1DM and 62 age and gender matched healthy controls underwent 3 T MRI of the brain. Volumes of the basal ganglia, amygdala hippocampus and thalamus were assessed with VBM. With this technique we show that in addition to cortical atrophy, T1DM differentially affects specific grey matter areas. In T1DM all subcortical grey matter structures (i.e. basal ganglia, hippocampus and thalamus) but the amygdala showed atrophy compared to healthy control subjects. Previous studies performed with lower field strengths in T1DM showed decreased hippocampal and thalamic volumes. Since the onset of T1DM is often in childhood, regional reductions may be likely to reflect changes that occur during brain development (14). It is interesting to speculate on the role of the amygdala in T1DM. It has a primary role

in controlling the cerebral regulation of stress response including the response to hypoglycaemia (15). Reduced activation of the amygdala has been found in subjects with T1DM and hypoglycaemia unawareness (16). Maintaining hypoglycaemia awareness and activation of the amygdala may therefore protect the amygdala from degeneration. However the clinical significance of this intriguing but challenging finding remains to be elucidated. Increasing awareness of the effects of T1DM on the brain is emerging; the necessity of brain research was recently incorporated into the report of unmet needs in diabetes research in Europe (<http://www.diamap.eu>). Future longitudinal studies in large patient populations are required to confirm these results and study underlying pathophysiology.

In addition to hypo- and hyperglycemia, a proposed underlying mechanism in target organ damage of the brain in T1DM, includes vascular disease. Increased aortic stiffness may contribute to brain damage by exposing the small vessels to high pressure fluctuations and flow in the circulation.

The study described in **Chapter 6** explored the association between aortic stiffness and diffusion tensor imaging (DTI) of the brain in T1DM. Forty-one patients with T1DM were studied and PWV was measured using a 1.5 T MRI scanner. DTI measurements were performed on 3 T MRI. This relatively new and validated MRI technique enables measurement of white matter microstructure and is based on diffusivity of water. We found that aortic stiffness is independently associated with reduced white matter integrity. Aortic PWV is a surrogate marker for arterial stiffness and has been shown to be an independent predictor for stroke and cerebral small vessel disease. To the best of our knowledge we are the first to show an association with white matter integrity in T1DM. These findings add further evidence to the hypothesis of a vascular contribution to brain damage. Large artery stiffness may trigger damage and remodelling of the microcirculation of the brain. Integrity of white matter tracts form the structural connections between brain regions. Previous studies have observed deficits in white matter microstructure in normal aging and cerebral small vessel disease. In addition decrements in white matter tract integrity have been shown in T1DM (17). However the clinical and prognostic implications of these microstructural changes and their relation with aortic stiffness have to be shown in future prospective studies.

The role of arterial stiffening in hypertension and concomitant chronic kidney disease is not completely elucidated. As arterial stiffening and impaired renal function often co-exist in hypertension, increased arterial stiffening may be related to both. During the cross-sectional study described in **Chapter 7** we included hypertensive patients with a normal estimated glomerular filtration rate, healthy volunteers and assessed the association between MR measured regional aortic PWV and renal function. We showed increased regional aortic stiffness of the proximal and distal aorta in hypertension com-

pared to healthy volunteers. Furthermore changes in renal function within the normal range are inversely related to arterial stiffness even in the absence of disproportionate stiffening. This relation did not differ between patients with hypertension and healthy volunteers and is present before the onset of decreased eGFR, proteinuria and macrovascular complications. These results stress the close link between aortic stiffness and the microcirculation. Future studies are needed to elucidate the implications of early arterial stiffening in hypertension on long-term renal function

With growing number of individuals with obesity and metabolic syndrome the implementation of lifestyle intervention becomes a major public health care challenge. **Chapter 8** evaluated the feasibility and effect of the implementation of a lifestyle intervention (inclusive of possible blood pressure treatment) among non-diabetic subjects with the metabolic syndrome in four primary health care centers. Subjects with the metabolic syndrome and insulin resistance were included (n=78) and offered a lifestyle program. Subjects with the metabolic syndrome without insulin resistance (n=311) were included and received a letter containing a set of lifestyle advices (non-intervention group). In the regression discontinuity design posttest scores were regressed on pretest scores with group as a covariate. For the effect on visceral obesity, glucose, lipid levels and blood pressure insulin resistance was added as a second covariate. The study showed that lifestyle intervention can be implemented in a primary care setting using existing facilities. Using the regression discontinuity design the intervention was shown to effectively reduce visceral obesity and lower systolic blood pressure. Despite a reduction in obesity the intervention did not affect insulin resistance levels possibly explained by the relative low intensity of the intervention and the use of a relative crude method for quantifying insulin resistance. Relatively few studies on lifestyle modification in metabolic at risk subjects have been performed in primary health care. Recently Bor et al. (18) explained the background theory and assumptions of the regression discontinuity design and introduced new possibilities for clinical practice. Although less efficient than a randomized controlled trial the design can be applied in existing care settings and can use existing data. Our findings may be more generalizable to daily practice than those of highly controlled randomized trials. Therefore this study may provide an effective strategy for clinical health care systems to meet the growing challenge of obesity and cardiovascular disease. Although frequently used in observational science, future studies are needed to examine the feasibility and robustness of the regression discontinuity design in medicine and clinical health.

CONCLUSIONS

Taken together the studies in current thesis contribute new information on biomarkers of end organ damage in the metabolic syndrome and diabetes. We have demonstrated that insulin resistance is a strong risk predictor for CVD and we provided a novel link between inflammation and ANGPTL4 expression. The imaging studies described in this thesis extend the knowledge on aortic stiffness and microvascular damage by showing an association with subtle microstructural deficits in T1DM. To improve our understanding of these microstructural deficits multicentre longitudinal studies that relate imaging parameters to cognitive performance are necessary. The predictive value of aortic stiffness would be highly interesting in this context because studies suggest an contributing role to microvascular damage. Furthermore regional and individual differences in response to an oral glucose load in MR assessed aortic stiffness were observed, which may open new future research paths possibly linking inter-individual variation in regional vascular response and CVD.

However given the cross-sectional design of the studies in this thesis, the biomarkers described provide no insight in changes over time. Longitudinal studies are needed to investigate whether they predict future events beyond established risk factors.

Hopefully future studies will show the usefulness of these biomarkers that will possibly select obese and overweight patients at high risk for CVD. Early detection of cardiovascular manifestations is highly relevant to allow better-targeted interventions to reduce CVD morbidity and mortality. Until then, the prevention of overweight and obesity worldwide remains the most effective strategy in reducing metabolic dysfunction and remains perhaps the biggest challenge for health systems world wide.

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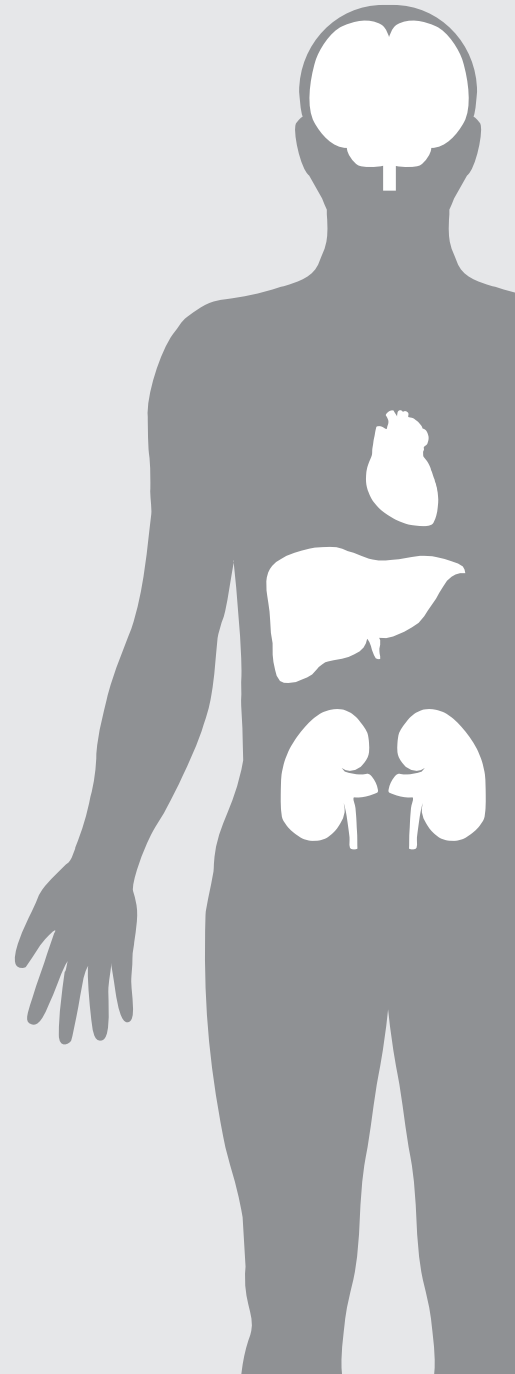
Chapter 10

Addendum

Nederlandse samenvatting

List of publications

Curriculum vitae



NEDERLANDSE SAMENVATTING

Hart- en vaatziekten (HVZ) behoren tot de belangrijkste oorzaak van overlijden in de Westerse wereld, waarbij overgewicht en obesitas het risico op HVZ aanzienlijk verhogen. Het aantal mensen met overgewicht neemt de afgelopen jaren sterk toe. Overgewicht gaat vaak samen met verminderde gevoeligheid van de weefsels voor insuline (insuline resistentie) waarbij zich uiteindelijk diabetes mellitus (DM) kan ontwikkelen. Insuline resistentie, gaat in vele gevallen van overgewicht gepaard met verhoogde concentraties in het bloed van glucose (hyperglykemie), vetten en cholesterol (dyslipidemie) en hypertensie. Deze clustering van risicofactoren voor HVZ wordt ook wel het metabool syndroom genoemd. In combinatie met laaggradige chronische ontsteking hebben deze factoren grote invloed op de ontwikkeling van cardiovasculaire eindorgaanschade.

Toegenomen vaatwandstijfheid van de aorta kan invloed hebben op de bloeddoorstroming van verschillende eindorganen waaronder de hersenen en nieren. Recent onderzoek toont aan dat toegenomen vaatwandstijfheid een belangrijke voorspeller is van HVZ. De elasticiteit van de aorta is namelijk van invloed op het omzetten van de polsgolf naar een meer constante bloedstroom. Bij een verminderde elasticiteit (en toegenomen vaatwandstijfheid) en daardoor een grotere polsdruk kan de perfusie van eindorganen worden beïnvloed. Aan de ontwikkeling van eindorgaanschade gaat jaren vooraf, dit biedt ruimte voor vroegtijdige preventieve interventies. Eén vijfde van de HVZ doet zich voor bij personen zonder traditionele risicofactoren. Het is daarom belangrijk om nieuwe voorspellers van HVZ en eindorgaanschade te identificeren.

In dit proefschrift worden biomarkers van cardiovasculaire eindorgaanschade bij patiënten met DM en het metabool syndroom beschreven. Biomarkers kunnen objectief gemeten en geëvalueerd worden en kunnen een onderliggende ziekte identificeren. Biomarkers kunnen verkregen worden via beeldvorming of middels bloedafname (biochemische biomarkers). In dit proefschrift zijn cross-sectionele studies met biochemische en magnetic resonance imaging (MRI) technieken beschreven. Daarnaast is het effect van een leefstijlinterventie op risicofactoren voor HVZ in de algemene populatie bestudeerd.

Hoofdstuk 2 is een systematisch literatuuroverzicht en meta-analyse waarin de associatie tussen de nuchtere bloedconcentraties van glucose, insuline, het Homeostasis Model Assessment of Insulin Resistance (HOMA-IR, een maat voor insuline resistentie) en de incidentie van HVZ is beschreven in personen zonder DM. Dit is de eerste meta-analyse die deze verschillende factoren direct vergelijkt in hun relatie tot HVZ. Er zijn 65 cohort studies en (nested) case-control studies met in totaal 516.325 proefpersonen geïncludeerd. Met behulp van een fixed-effect en random-effect model is het gepoolde relatief risico voor coronaire hartziekten, beroerte en HVZ berekend. Deze studie toont

aan dat in vergelijking met een toename in nuchter glucose of insuline het relatief risico op HVZ hoger is per standaarddeviatie toename in HOMA-IR. Bij gezonde mensen zorgt insuline ervoor dat te hoge glucosespiegels weer dalen, onder andere doordat insuline de opname van glucose in de weefsels stimuleert. Bij het ontstaan van insuline resistentie kan in eerste instantie gecompenseerd worden door een verhoogde uitscheiding van insuline door de bètacellen van de alvleesklier. Als dit tekort schiet ontstaan verhoogde glucosespiegels. Echter voordat de hoge glucosespiegels ontstaan is er al sprake van een gestoorde balans tussen insuline en glucose. Een marker voor insuline resistentie kan daarom van toegevoegde waarde zijn om het cardiovasculaire risico te voorspellen. Tevens speelt insuline resistentie een belangrijke rol in de vroege en gevorderde stadia van atherosclerose. Insuline resistentie lijkt het effect van insuline op de vaatwand te modificeren; anti-atherogeen in het geval van insuline gevoeligheid en atherogeen in het geval van insuline resistentie. Eerder onderzoek laat zien dat de toevoeging van het nuchter glucose aan de Framingham risico score, een veel gebruikt risico predictie model voor HVZ, resulteerde in een netto re-classificatie van 1.8%. Of de toevoeging van HOMA-IR aan predictie modellen, naast glucose, inderdaad zal resulteren in een betere inschatting van het cardiovasculaire risico moet nog bevestigd worden en zal bij mogelijk effect zorgvuldig moeten worden afgewogen tegen de met zich meebrengende kosten van het meten van HOMA-IR.

Hoofdstuk 3 evalueert of laaggradige systemische ontsteking een determinant is voor plasma angiopoietin-Like Protein 4 (ANGPTL4) concentraties bij diabetes mellitus type 2 (T2DM) en het metabool syndroom. In deze studie (n=334) zijn vier groepen bestudeerd: gezonde controles, patiënten met het metabool syndroom zonder systemische ontsteking, patiënten met het metabool syndroom met laaggradige systemische ontsteking en patiënten met T2DM. Initieel werden de deelnemers gegroepeerd op buikomvang en leeftijd. Plasma waarden van ANGPTL4, C-reactief proteïne (CRP) en andere metabole parameters werden bepaald. Deze studie laat zien dat ANGPTL4 toeneemt met stijgende inflammatoire status (gemeten met CRP) en toeneemt in de opeenvolgende groepen (gezonde controles < patiënten met het metabool syndroom zonder ontsteking < patiënten met het metabool syndroom met laaggradige systemische ontsteking < patiënten met T2DM). Concluderend zijn plasma ANGPTL4 levels gerelateerd aan systemische ontsteking. Wanneer alle personen werden gecombineerd werd een positieve correlatie gevonden tussen plasma ANGPTL4 en CRP. Om meer inzicht te krijgen in de causale relatie tussen ontsteking en ANGPTL4 expressie werd een *in vitro* experiment verricht waarbij werd onderzocht of stimuli voor ontsteking (Toll-like receptor 3 / 4 activatie) ANGPTL4 expressie in menselijke macrofagen verhoogt. Deze *in vitro* inflammatoire stimuli blijken de expressie en secretie van ANGPTL4 door macrofagen te verhogen. Tot nu toe werd gedacht dat de expressie van ANGPTL4 in verschillende

weefsels hoofdzakelijk werd gereguleerd door peroxisome proliferator-activated receptors (PPARs). Overeenkomstig de rol van PPARs, verhogen vrije vetzuren de expressie van ANGPTL4. Opmerkelijk genoeg vinden we in deze studie geen correlatie tussen vrije vetzuren in het plasma en plasma ANGPTL4 levels. De meest waarschijnlijke verklaring hiervoor is dat de studiedeelnemers en patiënten werden gegroepeerd op viscerale obesitas. De huidige bevindingen tonen een nieuwe relatie tussen ontsteking en de expressie van ANGPTL4. Toekomstig onderzoek zal nodig zijn om uit te wijzen welk mechanisme binnen cellen een toename van ANGPTL4 expressie in mensen veroorzaakt en of de ontsteking geïnduceerde toename van plasma ANGPTL4 oorzakelijk verband houdt met dyslipidemie.

Hoofdstuk 4 toont de waarde van MRI bij het bepalen van de regionale stijfheid van de aorta door het meten van de pulse wave velocity (PWV). PWV is een gevalideerde marker van vaatwandstijfheid en een belangrijke voorspeller van eindorgaanschade en HVZ. MRI is in staat om PWV nauwkeurig in meerdere locaties van de aorta te meten doordat MRI de werkelijk afgelegde afstand van de drukgolf in de aorta kan bepalen. In deze studie werd door middel van een placebo-gecontroleerde interventie gekeken naar het effect van een orale glucose belasting op de regionale PWV. De studie laat zien dat een orale glucose belasting leidt tot een regionale daling in PWV; deze daling werd alleen geobserveerd in de proximale maar niet in de distale aorta. Mogelijk reageren verschillende vaatbedden verschillend op vasoactieve invloeden zoals insuline. In eerdere studies werd reeds beschreven dat *in vitro* de bijdrage van stikstofoxide (NO) afhankelijk van vasodilatatie groter is in de centrale arteriën dan in de distale microcirculatie. Deze variatie in vasculaire reactiviteit is consistent met de regionale variatie in elastine versus collageen ratio van de aorta. Wellicht heeft postprandiale hyperinsulinemie minder effect op het distale deel van de aorta. Interessant in deze studie zijn de inter-individuele verschillen in de respons op de orale glucose belasting. Proefpersonen zonder significante verandering in PWV hadden een grotere buikomvang. Een eerdere studie beschreef reeds een verminderde insuline geïnduceerde daling in vaatwandstijfheid bij personen met overgewicht en verminderde NO synthese is beschreven bij patiënten met het metabool syndroom. Toekomstige onderzoek zou met name bij patiënten met DM de regionale effecten van hyperglykemie op verschillende segmenten van de aorta en de relatie met inter-individuele variatie in regionale verandering van PWV na glucose belasting tot onderwerp moeten hebben.

In **Hoofdstuk 5** worden de gevolgen van diabetes mellitus type 1 (T1DM) op het volume van corticale grijze stof met behulp van voxel-based morphometry (VBM) bestudeerd. Dit is een relatief nieuwe en geavanceerde MRI techniek waarmee subtiele grijze stof veranderingen vastgesteld kunnen worden. Voor deze studie ondergingen 62 patiënten

met T1DM en 62 op leeftijd en geslacht gegroepeerde gezonde controle personen een MRI van de hersenen. De volumes van de subcorticale grijze stof structuren bestaande uit de basale ganglia, amygdala, hippocampus en thalamus, werden onderzocht met VBM. Met deze techniek tonen we aan dat bij patiënten met T1DM alle subcorticale grijze stof structuren behalve de amygdala, atrofie vertonen. Eerdere studies toonden al een verminderd volume van de hippocampus en thalamus. Aangezien T1DM vaak ontstaat in de kindertijd is regionaal volumeverlies mogelijk een reflectie van veranderingen gedurende de ontwikkeling van de hersenen. Het is interessant om te speculeren over de rol van de amygdala in T1DM. De amygdala heeft een primaire rol in het controleren van de cerebrale regulatie van de stress reactie inclusief de respons op hypoglykemie. Verminderde activatie van de amygdala is gevonden bij patiënten met T1DM die lage glucose concentraties niet tijdig voelen aankomen (hypo-unawareness). Het behoud van een goede respons op lage glucose waarden en activatie van de amygdala zou daarom de amygdala mogelijk tegen degeneratie kunnen beschermen. Het klinisch belang van deze intrigerende bevinding moet nog worden onderzocht. De bewustwording dat DM ook effecten heeft op het brein neemt langzaam toe maar toekomstig onderzoek in grote patiëntpopulaties is vereist om resultaten te bevestigen en om de onderliggende pathofysiologie te onderzoeken.

Naast veranderingen in de glucosehomeostase, wordt een vasculaire component verondersteld in de pathogenese van hersenschade bij T1DM. Toegenomen stijfheid van de aorta kan invloed hebben op de perfusie van verschillende eindorganen door blootstelling van de microcirculatie aan hoge druk en aan druk fluctuaties. Mogelijk speelt toegenomen vaatwandstijfheid van de aorta een rol in het ontstaan van hersenschade. De studie in **hoofdstuk 6** beschrijft de associatie tussen vaatwandstijfheid van de aorta en diffusion tensor imaging (DTI) van de hersenen bij patiënten met T1DM. DTI is een relatief nieuwe en gevalideerde techniek die het mogelijk maakt om de microstructuur van witte stof te meten en is gebaseerd op de mate van diffusie van water. Bij 41 patiënten met T1DM werd de PWV van de aorta gemeten met een 1.5 T MRI. De beeldvorming van de hersenen werd met een 3 T MRI scanner verricht. Dit hoofdstuk demonstreert dat de stijfheid van de aorta onafhankelijk geassocieerd is met witte stof integriteit. PWV is een surrogaat marker voor arteriële stijfheid en is een onafhankelijke voorspeller van beroerte en microangiopathie van de hersenen. Voor zover bekend zijn wij de eersten die een associatie laten zien tussen witte stof integriteit en PWV in T1DM. Deze bevindingen suggereren dat vasculaire problematiek bijdraagt aan hersenschade. Stijfheid van de grote arteriën kan schade en remodelling van de microcirculatie van de hersenen bewerkstelligen. Witte stof banen vormen structurele verbindingen tussen verschillende hersengebieden. Eerdere studies toonden schade van de witte stof microstructuur bij normale veroudering en microangiopathie van het brein, echter de

klinische en prognostische implicaties van deze microstructurele veranderingen en de relatie met vaatwandstijfheid van de aorta zal uit toekomstige studies moeten blijken.

De rol van arteriële vaatwandstijfheid bij hypertensie en chronische nierschade is niet compleet opgehelderd. Vaatwandstijfheid en verminderde nierfunctie komen vaak gelijktijdig voor bij patiënten met hypertensie. Vaatwandstijfheid kan zowel bijdragen aan hypertensie als aan een verminderde nierfunctie. Disproportionele vaatwandstijfheid, dat wil zeggen een meer uitgesproken arteriële vaatwandstijfheid van het proximale systeem in vergelijking tot het distale systeem, kan de pulsatiele energie richting de microcirculatie bevorderen. Zo is microvasculaire schade van het brein geassocieerd met een meer verhoogde PWV van de arterie carotis ten opzichte van de PWV van de aorta. In **hoofdstuk 7** wordt de associatie tussen regionale PWV bij patiënten met hypertensie en gezonde vrijwilligers met een normale geschatte nierfunctie (creatinine klaring) beschreven. In deze cross-sectionele studie wordt aangetoond dat zowel de vaatwandstijfheid van de proximale als de distale aorta was toegenomen bij patiënten met hypertensie. Veranderingen in de nierfunctie waren omgekeerd gerelateerd aan vaatwandstijfheid, zelfs in de afwezigheid van disproportionele stijfheid. Deze relatie is zowel bij patiënten met hypertensie als bij gezonde vrijwilligers aanwezig en verschilde niet tussen de twee groepen. De resultaten benadrukken de relatie tussen vaatwandstijfheid van de aorta en de microcirculatie. Toekomstige studies zullen de betekenis moeten uitwijzen van vaatwandstijfheid voor de nierfunctie op lange termijn.

Met het toenemend aantal personen met obesitas en het metabool syndroom is de realisering van leefstijlinterventies een uitdaging voor de gezondheidszorg. **Hoofdstuk 8** evalueert de mogelijkheid en het effect van de implementatie van leefstijlinterventie (inclusief mogelijke bloeddrukbehandeling) bij patiënten met het metabool syndroom zonder T2DM in vier huisartsenpraktijken. Het effect werd geëvalueerd met behulp van het regressie discontinuïteitsdesign. In dit design wordt gebruik gemaakt van een bepaalde afkapgrens (discontinuïteit) die gebruikt wordt om een vergelijkbare behandeling en controlegroep te reconstrueren. Patiënten met het metabool syndroom en insuline resistentie ($\text{HOMA-IR} \geq 2$) werd een leefstijlinterventie programma aangeboden. Voor de groep zonder interventie werden mensen met het metabool syndroom zonder insuline resistentie ($\text{HOMA-IR} < 2$) geïnccludeerd. De interventiegroep ontving een brief met een aantal leefstijladviezen. Met behulp van het regressie discontinuïteit design werden post-test scores voorspeld met behulp van pre-test scores en groep (wel / geen interventie) als covariaat. De regressiecoëfficiënt voor groep reflecteert in dit design het effect van de interventie.

De studie demonstreert dat het mogelijk is om een leefstijlinterventie te implementeren in de huisartsenpraktijk met gebruikmaking van de bestaande faciliteiten. Met

behulp van het regressie discontinuïteit design wordt aangetoond dat de interventie leidt tot een afname in viscerale obesitas en een daling van de systolische bloeddruk. Ondanks de afname in obesitas laat de interventie geen effect op insuline resistentie zien. Mogelijk wordt dit verklaard door de relatief lage intensiteit van de interventie en het gebruik van HOMA-IR als relatief grove maat voor het kwantificeren van insuline resistentie. Er zijn relatief weinig leefstijlmodificatie studies uitgevoerd in de huisartsenpraktijk, terwijl de eerste lijn wel dicht bij de algemene bevolking en de dagelijkse praktijk staat. Recent werden de achtergrondtheorie en de aannames voor het regressie discontinuïteit design met de mogelijkheden voor de klinische praktijk geïntroduceerd. Ofschoon minder efficiënt dan een gerandomiseerd gecontroleerd onderzoek kan deze methode toegepast worden in de bestaande gezondheidszorg met gebruikmaking van bestaande data. Onze bevindingen zijn mogelijk meer generaliseerbaar naar de dagelijkse praktijk dan de bevindingen van gecontroleerde gerandomiseerde onderzoeken. Daarom kan deze studie een effectieve strategie bieden voor de gezondheidszorg om de groeiende uitdaging aan te gaan in de strijd tegen overgewicht en HVZ. Toekomstige studies moeten de mogelijkheid en robuustheid van het regressie discontinuïteit design in de geneeskunde en gezondheidszorg bewijzen.

Conclusies

De studies beschreven in dit proefschrift dragen bij aan nieuwe informatie over biomarkers en eindorgaanschade in het metabool syndroom en DM. We hebben aangetoond dat insuline resistentie een sterke risico voorspeller is voor HVZ en ontdekten een nieuwe link tussen ANGPTL4 en systemische ontsteking. De studies verricht met MRI dragen bij aan de kennis over vaatwandstijfheid van de aorta en microvasculaire schade door het tonen van de associatie met subtiele microstructurele witte stof schade bij patiënten met T1DM. Om kennis van deze microstructurele schade te verbeteren zijn multicenter studies nodig waarbij deze associatie wordt gerelateerd aan cognitief functioneren. Verder tonen de studies in dit proefschrift regionale en individuele verschillen in de effecten van een orale glucose belasting op vaatwandstijfheid. Meer onderzoek zal moeten plaatsvinden naar deze interindividuele variatie in regionale respons en de eventuele associatie met HVZ, met name in patiënten met het metabool syndroom en DM.

Het cross-sectionele design van de studies in dit proefschrift maakt dat er geen inzicht in veranderingen in biomarkers over de tijd is. Meer longitudinale studies zijn nodig om te onderzoeken of deze biomarkers HVZ kunnen voorspellen boven al bekende en veel gebruikte risicofactoren.

Hopelijk zal toekomstig onderzoek de bruikbaarheid van deze biomarkers tonen zodat patiënten met obesitas en overgewicht met een verhoogd risico op HVZ beter en eerder geselecteerd kunnen worden. Het vroeg kunnen voorspellen van cardiovas-

culaire schade is relevant voor interventies gericht op het reduceren van morbiditeit en mortaliteit. Tot die tijd blijft de preventie van overgewicht en obesitas wereldwijd de meest effectieve strategie in het verminderen van metabole dysfunctie en blijft dit misschien wel de grootste uitdaging voor gezondheidszorg systemen wereldwijd.

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CURRICULUM VITAE

Nathanja Tjeerdema werd geboren op 19 juli 1980 te Aalten. Zij behaalde haar VWO-diploma in 1999 aan het Bogerman College te Sneek. Na uitloting bij de numerus fixus voor de studie Geneeskunde studeerde zij Psychologie aan de Rijksuniversiteit Groningen. Tijdens de laatste fase van de studie Psychologie verrichtte zij onderzoek naar de behandeling van Body Dysmorphic Disorder en onderzoek naar rouwverwerking bij Post Traumatische Stres Stoornis. In augustus 2004 behaalde zij haar doctoraal met de twee afstudeerrichtingen klinische psychologie en neurobiopsychologie. Na drie keer te zijn uitgeloot begon zij in 2004 aan de zij-instroom voor de studie Geneeskunde te Leiden. In de wachttijd voor haar laatste co-schap verrichtte zij in 2006 als student onderzoek onder supervisie van dr. J.T.Tamsma. Na het behalen van haar doctoraalexamen in 2008 startte zij met promotieonderzoek op de afdeling Algemene Interne Geneeskunde en Endocrinologie & Metabolisme onder begeleiding van prof. dr. J.W.A. Smit, prof. dr. A. de Roos en prof. dr. P.C.N. Rensen. De resultaten daarvan zijn in dit proefschrift beschreven. In september 2012 is zij begonnen met de opleiding tot internist in het Bronovo Ziekenhuis te Den Haag (Opleiders Dr. Y.W.J. Sijpkens en Dr. E.D. Beishuizen). Inmiddels vervolgt zij haar opleiding in het LUMC te Leiden (Opleiders prof. dr. de Feijter en prof. dr. L.G. Visser).